

nature

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Little comfort for university teachers

Two and a half years ago the present Labour government of the UK introduced the first stage of a pay policy which is, of course, still in operation. In its first year the policy laid down that the maximum salary increase for anyone would be £312, and that those already being paid more than £8,500 per annum should get no increase at all. Succeeding stages have been equally tight-fisted. There can be little doubt that the policy has been effective in reducing the level of inflation from around the 25% per annum mark to the present 10%. But there have been obvious losers—the levelling nature of the policy has ensured substantial declines in real income for those paid above average, and the necessity for an arbitrary date of imposition in 1975 ensured that pay anomalies unresolved at that time remain unresolved. Nor in the present gradual thaw in incomes policy is there much comfort for those who live under the discipline of public sector cash limits or who are unable to negotiate deals on productivity. Into every one of these losing categories fall university teachers.

The 1975 clampdown occurred at a time when university teachers had gone to arbitration over their claim for the adjustment of parities with polytechnic staff and over a cost of living increase; they were left stranded. Everyone agrees that they have had a raw deal, and the salary scales speak for themselves: the lecturer scale starts at £3,333 per annum and professors can start as low as £8,100. At the low end of the pay scales, lecturers are widely able to claim social benefits that are intended for the poorer paid members of the community. More senior university teachers have seen their salary decline in real terms by up to 20% in the past few years, and by more when one includes 'fiscal drag'—the failure of tax rates and thresholds to move with the cost of living.

Even though there has been much hand-wringing by the government about the plight of university staff, it is not yet at all clear what the present negotiations between the Association of University Teachers (AUT) and the employers will yield. AUT, notably without industrial muscle, has said it is prepared to live with a rise that is consistent with the present 10% guidelines, but only with a firm timetable set for the elimination of the present anomalies. The union's expectation is that the anomalies would be removed by October of this year, and it has been handed a very fine precedent for such hopes by the firemen, who accepted an offer close to the guidelines only on the promise that they

would be well looked after in future years (assuming no change in the political complexion of the government). This form of agreement is not the sort of thing the government wants to repeat, however, and it has made the somewhat lame excuse that the firemen were unique because 'human life was at stake'. So university lecturers (who have actually increased their 'productivity' with significant increases in the student: staff ratio in the past few years, but who save no lives) are not held out much comfort.

The worrying thing in all this is not just the poor salary levels; it is a growing feeling that the Labour government is moving towards a perceptibly different style for universities without being entirely open about its intentions. A few weeks ago, the Commons debated the situation in the universities, including, of course, the pay problem, but much else besides. Two Labour MPs independently spoke about the need for greater public control of what goes on in universities, one going so far as to say that there was "a growing feeling in the country, particularly amongst younger Socialist politicians, that there should be a greater degree of public accountability for university funding". The Minister of State, Department of Education and Science, with special responsibility for higher education, Mr Gordon Oakes, did not exactly dissociate himself from such ideas, mentioning that the Labour Party had a discussion document on them, and going on to say that he hoped that in ten years "the university . . . will contain a 50-50 mixture—50 per cent coming from schools and 50 per cent who could be of any age".

From a minister, even to an almost empty House, this looks rather heavy stuff. Many, of course, will agree in general with his remarks about responsiveness but point out that a lot of universities, particularly those recently converted from colleges of advanced technology, scamper around quite a lot in an effort to meet the nation's wishes. But is he serious about a half-and-half university by 1988? Certainly there are demographic changes which will start to reduce the numbers of school-leavers after 1981, and certainly there is much scope for more continuing education. These most recent remarks, however, cannot but be unsettling at a time when university staff morale is not high. If ideas are indeed that advanced, the government should publish them, together with documentation, and give individual members of the university community a chance to have a reasoned discussion and give a sensible response. □



Saccharin: the risks and benefits

Bernard L. Cohen of the University of Pittsburgh argues that the decision on whether or not to ban saccharin should be taken in the light of risk-benefit analysis. The risk of cancer may be outweighed by the risks of obesity.

SCIENTIFIC decision-making in matters of public concern should be made on the basis of risk-benefit analysis; but in the controversy over the banning of saccharin there has been little evidence that this has been done. That controversy involves decision making by the general public, so it is important that the public understand the risks in some quantitative fashion.

Since the average person does not readily accept numbers, the best substitute is to compare the risks of saccharin with other risks with which members of the public are familiar. It is my purpose here to give both a risk-benefit analysis and to compare saccharin risks with other risks familiar in our daily lives. All calculations will assume a linear relationship between dose and effects as is customary in estimates of this type.

In the recent Canadian study of bladder-cancer patients (*Lancet*, 17 September 1977, page 578), a possible link was established between that disease and the use of saccharin such that if the United States population (2×10^8) were to ingest one diet soft drink each day throughout their lives, there would be an extra 1,200 bladder cancers per year. This implies a risk of 1,200 cancers per 7.3×10^{10} drinks, or one cancer per 6×10^7 drinks. There is ordinarily a time delay of 10 to 50 years between ingestion of a carcinogen and development of a cancer, so an average case would result in no more than a 20 year loss of life expectancy; thus, based on the results of the Canadian study, an average diet drink would reduce life expectancy by $20 \text{ yr}/6 \times 10^7$, or about 9 s.

To give a perspective on this number, smoking a single cigarette reduces life expectancy by 12 min (B. L. Cohen *American Scientist* 64, 550; 1977) so a diet soft drink is about 80 times less dangerous than a cigarette. From the above result (or from the original finding) it is straightforward to calculate that one diet drink per day throughout life causes a reduction in life expectancy, ΔL , of 2 d; or

$$\Delta L = 2 \text{ d} \left(\frac{\text{diet drinks}}{\text{d}} \right) \quad (1)$$

The benefits of diet soft-drinks result from their use in weight control by reducing calorific intake. Being overweight is well known to reduce life expectancy. In a rather old study, Pauling (*Proc. Natl. Acad. Sci.* 44, 619; 1958) analysed the data on this in a 1952 report of the Metropolitan Life Insurance Co. to obtain best fits to both a linear and quadratic relationship between loss of life expectancy, L , and overweight ($W - W_0$), where W is the weight and W_0 is the optimal weight. These were,

$$L = 17 \text{ yr} [(W - W_0)/W_0] \quad (2)$$

$$L = 36 \text{ yr} [(W - W_0)/W_0]^2 \quad (3)$$

Differentiating (3) and assuming that an average saccharin user would be at least 10% overweight and would weigh perhaps 160 lb gives

$$\Delta L = 0.05 \text{ yr/lb} \cdot \Delta W \quad (4)$$

Applying the same assumptions to (2) gives

$$\Delta L = 0.11 \text{ yr/lb} \cdot \Delta W \quad (5)$$

independent of the percentage overweight.

There is more recent data on this subject from the same source (*Metropolitan Life Insurance Co. Statistical Bulletin* 10; 1960). To quote typical figures, for a 45-year-old male with 150 lb optimal weight, weighing 170 lb reduces life expectancy by

1.5 years and weighing 200 lb reduces it by 4 years. This gives an approximately linear relationship with

$$\begin{aligned} \Delta L &= 0.08 \text{ yr/lb} \cdot \Delta W \\ &= 29 \text{ d/lb} \cdot \Delta W \end{aligned} \quad (6)$$

Since this is intermediate between (4) and (5) and is based on better data, I shall use (6).

An average person's body weight is related to his average daily calorie intake at about 1 lb per 14 calories per day. Multiplying this by (6) gives a change in life expectancy

$$\Delta L = 2 \text{ d/calories-per-d-intake} \quad (7)$$

From a comparison between (1) and (7) we see that diet soft drinks give a net benefit if one such drink reduces calorific intake by more than 1 calorie.

There seems to be no firm evidence on the amount by which diet drinks reduce calorific intake (or body weight). A non-diet drink has about 100 calories, so if all other things were unchanged, substituting diet for non-diet drinks increases life expectancy by 100 times more than the cancer risk reduces it. This is perhaps an extreme assumption, but it would be very difficult to prove that it over-estimates the reduction in calorie intake from diet drinks by a factor of 100. Unless this is done, there is no proof that the risk from diet drinks is greater than their benefits, and in fact it seems most likely that the opposite is the case.

If, on the other hand, we ignore the foregoing discussion and assume that there are no benefits from use of saccharin, it becomes important to understand the risks. We have already noted that ingesting a diet drink is 80 times less dangerous than smoking a cigarette, and we discuss here some other comparisons.

Perhaps the simplest risks to quantify are those due to automotive traffic. Approximately 8,000 people per year are killed in crossing streets in the United States. If the average American crosses five streets per day (3.5×10^{11} crossings per year), the risk per crossing is $8,000/3.5 \times 10^{11}$ or 2.3×10^{-8} . The risk from a diet drink is $1/6 \times 10^{-7}$, or 1.6×10^{-8} . Thus an average street crossing is about as dangerous as ingesting a diet drink.

Riding in automobiles involves a death risk of 2×10^{-8} per mile; thus a diet drink is as dangerous as one mile of automobile travel. Using a small car rather than a large car approximately doubles the risk. So purchasing a small car rather than a standard size car is at least ten times as dangerous (assuming 30 miles per day average driving) as ingesting one diet drink per day during the period of ownership.

Perhaps the best understood cancer risk is that of women not having an annual test for cervical cancer. The mortality risk from this neglect is about 6×10^{-7} per day, about 30 times higher than the risk of ingesting one diet drink per day.

The risk of one diet drink per day throughout life is readily calculated, using the Canadian study results, as $(1200/2 \times 10^8)$ risk per yr $\times 70 \text{ yrs} = 4 \times 10^{-4}$. Statistics show that the cancer risk varies considerably from one area of the country to another. For example, the risk is about 0.19 in New England compared to 0.15 in the south-east which has a similar age distribution. Thus moving from the south-east to New England involves 100 times greater cancer risk than ingesting one diet drink per day throughout life.

Approximately 7,000 Americans die each year due to fires, and it is estimated that about half of these could be saved if people would install smoke alarms in their homes (cost—about \$5 per year). Failure to install a smoke alarm is thus as dangerous as ingesting three diet drinks per day.

An endless list of comparisons could be put forward, but we have offered here a few examples that may be useful in helping understand the risk of saccharin. □

Alternatives to sugar

The search for an ideal non-nutritive sweetener is almost a century old.

K. J. Parker outlines its history

SWEETNESS has long been known as a property possessed by many substances other than sugar, although their use as sweeteners has largely been confined to pharmacy, or precluded by toxicity. For example, chloroform, which was discovered in 1832, is still used for sweetening lozenges and linctus. The intensely sweet taste of liquorice root was known to the ancient Egyptians, an extract having been found in the tomb of King Tut (1500 B.C.). Although its strong liquorice flavour and pharmacological activity today limit its wider application as a sweetener, it is used in pharmacy, in some confectionery, and as a flavour potentiator.

The commercial development of artificial sweeteners dates from the discovery of saccharin in 1879, by C. Fahlberg and I. Remsen, followed by its manufacture five years later. In that year, the intense sweetness of *para*-ethoxyphenylurea was discovered by J. Berlinerblau, and within ten years, this compound was being marketed under the name of *dulcin*.

By the end of the last century sugar had become accepted as a basic food item, and its use and ready availability were taken for granted. Saccharin provided an alternative sweetener to sucrose, for use in times of sugar shortage, as a cheap substitute for carbohydrate sweeteners, or for inclusion in sugar-free diets. Its slightly bitter flavour and metallic after-taste, however, contrasting with the pure sweet taste of sucrose, reduces its acceptability. Consequently, the production of saccharin, which in Germany had reached 100,000 lb/year by the turn of the century and increased sharply during the two world wars, remained fairly static in Britain and Europe for the first half of this century.

Before 1940, inadequate nutrition had been a major concern of world health authorities. A decade later it began to be more widely appreciated that the converse—excessive or unbalanced food intake—could be equally injurious to health, possibly contributing to heart diseases, obesity and metabolic disorders. Since the need for essential dietary factors—vitamins, minerals, amino acids—was recognised, the emphasis for control of nutritional intake inevitably focused on carbohydrates and especially on sugar. Although the polemics of this particular view are still being debated, the substitution of sugar in the diet by a non-nutritive alternative has already

proved to be a painless and popular means of reducing carbohydrate—and hence, calorie—intake.

The demand for saccharin and its particular shortcomings meant that the market was ready for a more acceptable alternative. This was provided by a new sweetener, *N*-cyclohexylsulphamic acid, or cyclamate, discovered in 1937 by L. F. Audrieth and M. Sveda. In spite of its having only less than one tenth the sweetness of saccharin, the flavour of cyclamate was considered to be singularly pleasant and free from after-taste. Admixture of 10% saccharin effectively doubled its sweetness without introducing undesirable after-taste.

Legislation on sweeteners

The use of saccharin-cyclamate sweeteners, particularly in soft drinks, which currently accounts for some 70% of the synthetic sweeteners consumed, increased tenfold between their introduction and 1968. By then, concern was mounting over the potential hazard of unrestricted consumption of artificial sweeteners, especially by children, as a consequence of the widespread use of saccharin-cyclamate in non-dietetic foods and beverages.

A major manufacturer, Abbott Laboratories, had spent an estimated \$1 million on establishing the safety of this sweetener. Ironically, their disclosure that massive doses of cyclamate fed to rats was associated with the development of malignant bladder tumours in some of the test animals led in October 1969 to an immediate ban on the use of cyclamates in the United States. Great Britain, Canada and several other countries followed with equivalent legislation, though the restriction was not universal. Saccharin the only remaining permitted artificial sweetener, was subsequently removed from the Food and Drug Administration (FDA) list of food additives generally recognised as safe (GRAS list). Finally, in March of this year, following the results of at least six separate studies on rats in which cancerous tumours were positively identified, the use of saccharin in diet foods, cosmetics and non-prescription drugs was ordered to be withdrawn in the United States within two years, under the controversial 1958 Delaney amendment to the 1938 Food, Drug and Cosmetic Act. The Delaney clause makes mandatory the banning of the food use of any substance shown to induce cancer in any animal under any conditions.

It was against this background of an increasingly urgent demand for a safe non-nutritive sweetener that research into the chemical basis of sweetness was boosted in the late 1960s. In spite of the extensive research into the physiology and psychology of taste perception, however, it is still not possible to predict the chemical structure of an ideal non-nutritive sweetener, or even that a substance will be sweet. Many types of compound are known to taste sweet, but in every class the initial discovery of sweetness was accidental. There seems to be no common molecular property which can be used to predict the ability to elicit a sweet taste.

Theoretical models have been advanced for the molecular basis of sweetness, the most comprehensive being that of Robert Shallenberger of Cornell University. In its most recent form, his model accounts for the perception of sweetness in terms of a three-point interaction with the taste receptor protein, involving a proton donor, a proton acceptor and a lipophilic group sited at the points of a precisely specified triangle. In spite of the ingenious explanation of the sweetness of known compounds, however, not all compounds fitting the model are sweet and the theory cannot be used to predict or construct radically new sweeteners.

Nature and nurture

Research into new sweeteners thus takes the form of molecular variations on a known structural theme. Alternatively, intensely sweet substances of natural origin can be sought and characterised. Either approach, however, is ultimately subject to the requirement that the candidate sweetener should meet the increasingly rigorous toxicological safety standards imposed by the public health authorities, before it can be permitted for use as a food additive. Since toxicity cannot be predicted on the basis of molecular structure either, the search is inevitably speculative.

Since the ban imposed on cyclamate, Abbott Laboratories have been continuing to sponsor research into the toxicology and safety of cyclamate, and in November, 1973, petitioned the FDA for its reinstatement on the grounds of need and in the light of further experimental evidence. It is argued that the single piece of evidence indicting cyclamate was not substantiated by subsequent studies. With the

eventual withdrawal of saccharin there will be no permitted sweetener on the US market, a situation in which the FDA may be obliged to reconsider rescinding the earlier ban on cyclamate, though so far the Abbott petition has not been allowed.

New synthetic sweeteners

Several candidate sweeteners are being developed, but very little published information is available. The market is highly competitive, and no wonder for production of a multi-billion dollar sweetener is the prize.

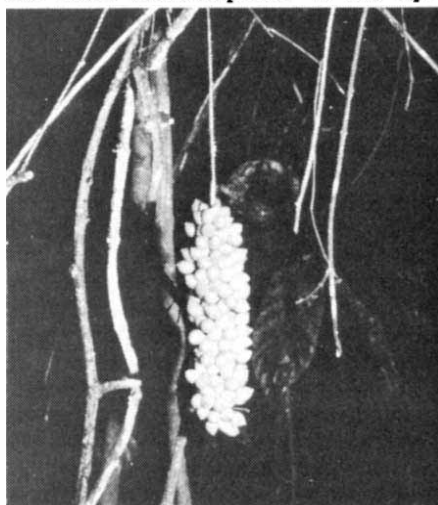
In addition to cyclamate, two other non-calorific sweeteners, Aspartame and β -neohesperidin dihydrochalcone, have been under consideration by the FDA, though neither has yet been declared wholly acceptable. The intense sweetness of the methyl ester of the dipeptide α -L-aspartyl-L-phenylalanine was discovered by James Schlatter working in the G. D. Searle & Co. laboratories and reported by R. H. Mazur, J. M. Schlatter and A. H. Goldkamp in 1969, though the compound had been first synthesised three years previously within ICI. Searle filed a patent for the use of this compound as a sweetener, while ICI patented the cyclohexyl analogue at almost the same time.

Since this discovery, several hundred closely related compounds have been synthesised, many of which have proved sweet. However, Searle decided to develop and market the original dipeptide methyl ester under the name of Aspartame, in conjunction with the Japanese Ajinomoto Company, which had independently developed a cheaper synthetic route. The possible formation of the cyclic self-condensation product diketopiperazine, formed in soft drinks on storage, for example, resulted in Searle voluntarily withholding the product from the market, even though qualified approval had been granted by the FDA in 1974 for the use of Aspartame as a table sweetener. Then in December, 1975, the FDA rescinded its earlier order indefinitely, pending further review of the evidence on safety, in spite of the removal of earlier fears on the carcinogenic properties of diketopiperazine.

In a study of the bitter principles of citrus fruit rind, R. M. Horowitz and B. Gentili discovered that the bitter flavanone naringin from grapefruit peel could easily be converted into the corresponding dihydrochalcone which by contrast was found to be intensely sweet, a property shared by a number of related dihydrochalcones. Of these β -neohesperidin dihydrochalcone was selected for toxicological and metabolic studies by the Southern Regional Laboratory of the US Department of Agriculture. The pilot plant production was also developed making possible its

manufacture in ton quantities when required. Although its low solubility in water and the delay in taste perception make this sweetener unsuitable for many applications, once granted food additive status it could be used for sweetening chewing gum, toothpastes, pharmaceutical preparations, etc where such properties are not a disadvantage.

A new class of sweeteners, the oxathiazinone dioxides, was reported in 1973 by K. Clauss and H. Jensen of Hoechst. These, like saccharin and cyclamate, contain the sulphonamide group and a lipophilic part. The β -methyl derivative, which has been reported to have the best flavour profile, is being developed by Hoechst under the name Acetosulpham. It has ap-



Wild serendipity berries

proximately one third the sweetness intensity of saccharin.

Although several amino acids are known to taste sweet, it was discovered accidentally, by Kornfeld of Eli Lilly and Co., that some 6-substituted D-tryptophan derivatives are intensely sweet. Of these, 6-chlorotryptophan, reported to be approximately 1,000 times as sweet as sucrose, has been selected for development as a sweetener by the company.

A systematic study of the relationship between taste and chemical structure of a series of terpene oximes and related compounds has been undertaken by a research team at Stanford University led by E. M. Acton. The intense sweetness of the α -syn-oxime of perillartine was first reported in 1920 by Furukawa in Japan, although this compound is unsuitable for use as a sweetener owing to its low water solubility. However, a closely related oxime has been proposed as a suitable candidate sweetener by the Stanford group.

Since the early discovery of the intense sweetness of alkoxyaromatic amines—examples of which are Dulcin and, more recently, the deep yellow P 4000, used briefly as a sweetener in

Holland during the war—numerous intensely sweet arylamines have been described. These compounds are generally toxic, and are thus unlikely to be selected for further development. However, A. Zaffaroni, of the Dynapol Corporation of America, has conceived the idea of linking a sweet molecule to a non-absorbable, inert polymer in such a way that the taste interaction of the sweet part is unaffected. Not being metabolised, the sweetener cannot manifest systemic toxicity and is non-calorific.

Why sugars and polyhydric alcohols reach a limiting level of sweetness with sucrose, while non-hydroxylic compounds of similar molecular weight can be several orders of magnitude sweeter



Thaumatococcus plants

can be explained by the absence of a lipophilic group from the sugar molecule. Pursuing this theme, Michael Lindley and Gordon Birch of the National College of Food Technology, Reading University, prepared several methyl ethers of sucrose but none was found to be particularly sweet. This approach was vindicated, however, with the discovery by Leslie Hough and S. P. Phadnis of Queen Elizabeth College, London, of the intense sweetness of tetrachlorogalactosucrose, while working in collaboration with Riaz Khan of Tate & Lyle, on deoxy- and halodeoxysucrose derivatives. They subsequently showed that sweetness reaches a maximum in 1',4,6'-trichlorotrideoxygalactosucrose, found to be approximately six hundred times sweeter than sucrose. It has a pure sweet flavour closely similar to that of sucrose. Although it has been shown not to be toxic to rats and to be non-calorific, its suitability as a non-nutritive sweetener has yet to be established.

Natural sweeteners

The occurrence of high-intensity sweeteners in nature has long been recognised, many having been used traditionally for generations: for ex-

ample, glycyrrhizin from the roots of the liquorice plant, the fruit of the South Chinese Lo Han Kuo and the leaves of the Paraguayan shrub *Stevia rebaudiana*. Stevioside, a triterpene glycoside, which is readily extracted from the leaves of *S. rebaudiana*, is being produced on a commercial scale in Japan by the Toyo Menka Karisha Company. Stevioside, which is approximately 300 times sweeter than sucrose, is not a permitted food additive elsewhere.

More recently the serendipity berry (*Dioscoreophyllum cumminsii*) was identified in a broad survey of sweet fruits undertaken by George Inglett and J. F. May, Department of Agriculture, in 1968. Its berries are intensely sweet owing to the presence of the protein monellin which is approximately 2,000 times as sweet as sucrose. A similar protein, thaumatin, has been isolated from the fruit of a West African plant *Thaumatococcus daniellii* by H. van der Wel and K. Loeve of Unilever. This has proved to be the sweetest substance known, being

around 4,000 times sweeter than sucrose. The extraction, purification and properties of both monellin and thaumatin have been studied extensively, in particular by Tate & Lyle, who are able to produce thaumatin in kilogramme quantities. However, the toxicology of thaumatin has not been fully evaluated, though, being a vegetable protein which has been consumed for generations, it seems unlikely that it would have harmful side-effects.

Similar considerations would seem to apply miraculin, the glycoprotein extracted from the berries of the bush *Synsepalum dulcificum*, also native to West Africa. An extract of this fruit, which has the property of tasting intensely sweet only in the presence of acids, was marketed for a time by the Miralin Corporation of America who established extensive plantations in several countries to produce the extract. However, FDA approval for this product was denied in 1974 pending toxicology testing, with the consequence that it is not now commercially available.

Naturally occurring sugars and sugar alcohols, such as fructose, xylitol and maltitol, are also being evaluated as alternative sweeteners, though since they are metabolised, they cannot be regarded as non-calorific. Xylitol, produced from birch wood, is available at ten times the price of sugar. It is finding use as a sweetener in chewing gum, though it is not a permitted sweetener in Britain and, following recent evidence of potential carcinogenic activity, may be prohibited in the US.

In spite of the intense interest worldwide in developing alternative sweeteners, the ideal non-nutritive sweetener—water soluble, chemically and thermally stable, of pure flavour, non-toxic, and of high sweetness intensity—has not yet been discovered and may still be some years off. In the meantime a less satisfactory compromise seems inevitable. □

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No soft options in Carter's energy research policy

ENERGY policy was a major plank in Jimmy Carter's campaign platform during the 1976 Presidential election. In a year when the rising cost of imported oil was causing increasing economic concern, and the late E. F. Schumacher was telling capacity audiences that "Small is beautiful", Carter promised that, if elected, he would shift the emphasis of US energy technology away from a prime dependence on nuclear fuels and towards non-nuclear alternatives, in particular renewable resources such as solar power.

But like many other campaign promises—for example that to reduce the budget of the Defense Department, now scheduled for a 9.4 per cent increase to \$117.8 billion in fiscal year 1979—Carter's energy plans have, in the face of established institutional positions and interests, undergone considerable revision in his first year of office.

The President has maintained his firm stand on the dangers of proliferation resulting from the production of plutonium by fast breeder reactors, in particular demanding the closure of the Clinch River liquid metal fast breeder reactor at Oak Ridge, Tennessee.

But the administration is still pursuing a vigorous nuclear policy through other options, including possibly a major design project for a thorium-

cycle reactor. And already last year, following, in particular, moves to speed up the licensing of new reactors, Carter's earlier claims that he would turn to nuclear energy only as "a last resort" were beginning to wear thin.

Further confirmation that energy policy has changed little comes from analysis of the Department of Energy's proposed research and development budget for the fiscal year 1979. In presenting this to Congress two weeks ago, President Carter has suggested that the overall level of energy R&D remain roughly static at \$2.7 billion.

The budget contains considerable increases in commitments to research on synthetic fuels (another area in which campaign promises seem to have been forgotten). In contrast, resources for research into renewable energy sources show little change. It is proposed, for example, to raise the budget for windpower research from \$37 million to \$41 million; and solar power research, which has grown from \$4 million in 1974 to \$193 million in 1977 and \$303 million last year, will increase only to \$309 million. The increases in these two topics scarcely keep up with the expected increase in the cost of living.

There are, admittedly, some important shifts in philosophy reflected in the budget proposals. Perhaps the

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The inauguration of the US National Aeronautics and Space Administration's windmill in New Mexico

most significant is a hefty 27% increase in funds for conservation R&D, under which funds for research on topics such as motor efficiency and energy storage will be increased from \$301 to \$381 million.

In addition, the proposed closure of the Clinch River fast breeder project, the subject of a furious political battle last autumn, after which Carter vetoed the authorisation bill and is yet to decide what to do with a supplemental appropriation of \$80 million passed by Congress, would save \$150 million, creating an apparent swing in emphasis from nuclear to non-nuclear technology.

But apart from the Clinch River project, there has been little change in relative priorities. Indeed if the Clinch River funds are extracted from the 1978 budget figures, the proportion of R&D expenditure going to other nuclear projects actually increases between 1978 and 1979, from 45.8 to 46.4 per cent of the total for research and technology development.

There is therefore still a strong continuation of pre-Carter policy directions. Dr John Deutch, who recently moved from chairman of the department of chemistry at the Massachusetts Institute of Technology to become director of the Office of Energy Research, told a House Subcommittee last week that "the strategic approach to our nation's energy R&D efforts has been evolving over the last several years".

But if there has been no obvious shift in priorities, there has been a significant change in strategy. In presenting the President's proposed R&D budget two weeks ago, Dr Frank Press, director of the Office of Science and Technology Policy, said that the administration was trying to shift the emphasis of R&D funding away from development projects—considered the responsibility of private industry—towards more basic research.

Dr Deutch shares this strategy, although he puts it slightly differently. In an interview with *Nature* last week he pinpointed the problem in energy research as lying not at the level of basic science, in which the department's position is relatively strong, but at an intermediate point in the R&D spectrum which he identifies as "advanced development work". In this he includes fields such as geology, and chemical and process engineering.

Dr Deutch also stresses the need to maintain flexibility in research programmes, developing a "hedged" strategy and keeping options open, so that the department can respond quickly both to new research potentials, and to changing parameters affecting energy supply.

In the nuclear field, for example,

one criticism of the Clinch River project has been that its massive funding has required putting too many eggs into one basket, and that the project was an attempt to move too fast in a single direction when revised predictions of future energy demand are indicating that the speed may be premature.

In contrast, the administration is suggesting that a close look should be taken at a wide range of options in the nuclear field, and at an earlier stage than project development. Thus within an overall R&D budget that is virtually static, funding for "basic energy sciences" is being increased by 20 per cent from \$177 million to \$212 million, and for nuclear research and applications by 22 per cent from \$227 to \$278 million. 'Advanced technology and assessment projects', which will involve a mechanism for monitoring and providing a critique of R&D programmes, will be increased almost three-fold, from \$8 million to \$21 million.

One alternative that President Carter is known to be particularly enthusiastic about is the thorium cycle reactor which, by using the thorium-uranium conversion process rather than the uranium-plutonium process, would be much safer than the conventional fast breeder.

Carter's enthusiasm for the thorium cycle is not shared by the environmentalist lobby, many of whom feel that it still fails to meet adequate safety criteria, at least in comparison with once-through light water reactors. But the Department of Energy is expected to announce soon a major \$15 million project for the conceptual design of a 650 to 900 megawatt reactor, to fill the programme gap left by terminating the Clinch River project; and the thorium cycle is a high contender for this new design.

Dr Deutch said last week that he felt

there was still not sufficient stress on solar research and development. But in line with the administration's general approach to R&D, funds for "production, demonstration and distribution" of solar energy supply are being reduced from \$87 million to \$64 million in 1979.

The decision to limit the growth of solar research funds has not been a popular one in Congress, where solar energy, with its apparent promise as a pollution-free, virtually infinite resource, carries a strong and populist appeal. Pointing out in the House of Representatives that the cutbacks in the solar energy programme were more than the salary increases requested by the Department of Energy, Representative Louis Frey Jr of Florida claimed that the budget could be characterised as "one giant step backward in energy research and development."

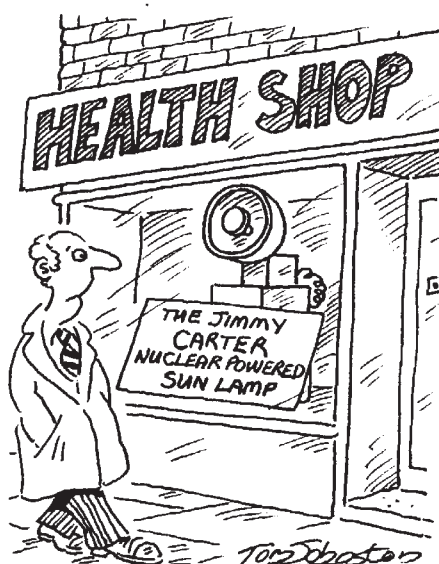
There will be a powerful campaign to increase solar energy's share of the R&D budget when the latter is debated in committee. And the campaign will receive added fuel from a shortly-expected report from Congress' General Accounting Office, which is thought to suggest that as much as \$600 million—almost twice the amount proposed by the administration—could be fruitfully absorbed into existing and proposed solar energy research projects.

In itself, the apparent conflict between the administration and Congress is not too important. In areas known to have enthusiastic support on Capitol Hill, the administration is often claimed to feel safe in going for a low budget knowing that it will be increased, and that it can put in higher bids in areas which Congress traditionally cuts back.

But in this case the stakes are higher than usual. President Carter has publicly asked to be judged by the success of his energy policy; hence his frustration at the stalemate on energy legislation, and the important symbolic role that it has taken on. However, in cutting back on solar energy research and continuing to emphasise nuclear options, whatever the administrative and scientific logic, he stands to lose the confidence of precisely those whose support he needs to push through his whole energy policy.

As one environmentalist who has been closely involved with recent debates said last week, in words that have been echoed across a range of issues: "I'm obviously disappointed that Carter's going back on his campaign promises, but I'm more disappointed that he won't admit what he's doing, that he won't come out and say that he has changed his mind."

David Dickson



Bangladesh sets up institute of nuclear agriculture

THE Vice-President of the Bangladesh Republic officially inaugurated the Institute of Nuclear Agriculture (INA) last month. This Institute, set up in collaboration with the International Atomic Energy Agency (IAEA), is the fourth large one of this kind to be built. The others are in Yugoslavia, India and Brazil. The Institute has been established in the premises of Bangladesh Agricultural University with the objective of identifying and solving the basic agricultural problems of the country through interdisciplinary approaches employing both nuclear and conventional techniques.

Agriculture is the mainstay of the Bangladesh economy. Farm products alone account for about 55% of the Gross Domestic Product (GDP), so the importance of boosting productivity can hardly be over-emphasised at a time when the country is facing the joint problems of food shortage and a fast growing population.

The initial research programmes of the Institute are in plant physiology, plant genetics, soil science, and ento-

mology, with emphasis on improving varieties of rice, jute, wheat, pulses, tomato and oil seeds. INA has already achieved some encouraging results in evolving new rice varieties, such as IRRATOM-24 and IRRATOM-38 through irradiation technique. They are capable of higher yield and early maturing than mother variety IRRI-8. Similarly, the two new strains of jute known as ATOMPAT-8 and ATOMPAT-38 have been found superior to the mother, D-154.

Mr Hellio F. S. Bittencourt, Deputy Director General of IAEA, addressing the inaugural ceremony said that the application of nuclear techniques in agricultural research was by no means a luxury for the developing countries; it was the base proven method for solving certain practical problems of agriculture.

The Swedish International Development Agency (SIDA) has provided a grant of over one million US dollars towards the cost of equipment, advisory service and fellowships, IAEA being the executive agency.

M. Kabir

Less paper, more money for US scientists

DR FRANK PRESS, director of the US Office of Science and Technology Policy, has launched a major campaign to encourage Government agencies to reduce the amount of time that university scientists receiving federal support are required to spend on paperwork.

Speaking in Washington last week, Dr Press said that the money that could be saved in this way might amount to millions of dollars for every university.

"One of the goals of this administration is to make universities perform better the basic research which the government subsidises. One way of reducing paperwork and overheads, for example, would be to award longer-term grants, so that scientists do not have to be writing new research proposals every three months or so."

Dr Press said that the Department of Health, Education and Welfare, which is responsible for the research carried out by the National Institutes of Health, is already looking at ways in which it can help universities by reducing paperwork and streamlining regulations about grants.

"I have also written to NASA (the National Aeronautic and Space Administration) and to NSF (the National Science Foundation) asking them what their plans are on this," he said.

Dr Press said that, under the current legislative framework, the administration could not guarantee support for a particular research project over a two or three year period, since the research budget had to be approved annually by Congress.

"However we can say to someone that we intend to support his work for a given number of years, and that if he or she sends in a report on the progress of his work at the end of each year which is satisfactory, we will agree to further funding." The Science and Technology committee of the House of Representatives is already looking into the possibility of multi-year authorisations as a way of stabilising the research-funding process, he said.

Dr Press also said that although there was little support in the administration for setting up a Department of Science, there was growing interest in establishing a Department of Technology and Industrial Development.

"Many countries, such as Germany, England, France and Australia, already have such ministries. This could be an interesting direction for the administration to go, particularly since there is an increasing sensitivity at Cabinet level to the problems of industrial innovation."

David Dickson

The facts about Kosmos-954

RECOVERY of the fragments of Kosmos-954 is proving, not surprisingly, a lengthy operation, and although some sizeable pieces have apparently been located and taken for examination to White Shell, Manitoba, it may be some considerable time before any hard facts are available about the type of satellite and reactor actually involved. Although the figure of 50 kg U-235 has been tossed around fairly freely, this is simply an assumption the reactor involved was of *Romashka* type. This is the only Soviet reactor of comparable type ever described in open publications; accordingly, it is the only basis for speculation. The *Romashka* has a 40 kW thermal yield and was, indeed, designed for space use, and assuming that such reactors are still used aboard certain Soviet satellites, it is possible to work out tentative figures for radiation hazards for this, and possible future, satellite crashes.

Three scenarios have been discussed among the experts. The first—break-up of the satellite in the stratosphere—requires the use of additional data. This comes from the US Navy SNAP-9A, powered by a plutonium source, which disintegrated before entry into orbit in 1964. On this basis, assuming an initial charge of 50 kg U-235—and 100 days in orbit before disaster, one arrives at the comforting figure that six months after the incident the residual radioactivity would be of the order of 10^{-9} of the International Commission for Radiological Protection safety level. (This is the concentration of airborne radioactivity that could be breathed 24 hours a day without harm.)

For break-up on impact, the downwind distance to which the UK Medical Research Council emergency reference levels extend works out at the order of a few km. (This means in effect that beyond this distance the hazards of mass evacuation are considered to be greater than the risk from radiation). For impact in one piece, if no chain reaction occurred, initial radiation levels would be expected of 1 rad/h at 200 m from the site, 100 millirad at 500 m, 10 millirad at 1500 m. Had the impact occurred in a populous area, evacuation would have been necessary up to 2 km, if no intervening shielding (e.g. masonry) were present. Chain reaction is unlikely, since the mass of uranium used in such satellites is normally subcritical, and special methods, e.g. a reflector, must be used to keep sufficient neutrons within it to maintain a chain reaction. Such a reflector would virtually certainly become

detached and destroyed during uncontrolled descent.

However comforting or worrying these figures, there is no doubt that the gloomy prediction of Ecclesiastes that "they shall be afraid of that which is high" has taken on a new and urgent meaning. President Carter has already expressed his willingness to be a party to a treaty banning the use of reactors from orbit. For the Soviet side, Academician Leonid Sedov has said in a TASS interview on Soviet radio that the errant satellite was in no way the "flying nuclear bomb" which some "absurd rumours" would make it. There were not and could not be any weapons aboard, he said, and those who spread such tales were trying to undermine the basic principles of international cooperation in the exploration and peaceful use of outer space.

In spite of Sedov's reassurances, it seems likely that Kosmos-954 did have certain military potentialities. Technically, it was a low orbit satellite for ocean surveillance. The presence of a reactor aboard (as opposed to a weaker isotope source) implies the use of powerful radar for monitoring ship movements, which would suggest that the terms of reference of the international agreements on the peaceful uses of both space and atomic energy were being somewhat stretched. Any future agreement on reactors in orbit might well have to re-examine the definition of 'peaceful' in this context.

Doubtless as a further reassurance, Sedov presented the official Soviet view of the accident: a sudden depressurisation of the satellite while beyond the range of Soviet tracking facilities. Since depressurisation was so rapid, it is assumed that Kosmos-954 collided with "some other object of natural or artificial origin".

Strangely, Sedov gave the significant date as January 6—the date of the "depressurisation". Yet according to US observers, the satellite was already misbehaving on December 17. The two reports are not incompatible—if the satellite was already out of control, it might well have come into contact with some other artificial object or debris which it normally should not have encountered. This, however, is not Sedov's story: he maintains that the on-board systems became inoperative only as a result of the January 6 impact—one more minor mystery to add to the story of Kosmos-954.

One fact, however, has already emerged quite clearly—the cost of the search and recovery operations. According to Canadian Prime Minister Pierre Trudeau, by last weekend this had reached one million dollars and was rising daily.

Vera Rich

Britain's big science on the baseline

AFTER four months in office, Professor Geoffrey Allen, Chairman of the UK's Science Research Council (SRC), is pleasantly surprised that the Advisory Board for the Research Councils (ABRC) is not as hostile to basic science as he had been led to believe by his predecessor, Sir Sam Edwards.

Professor Allen, speaking at a British science writers' luncheon, said that in spite of the proposed 1.7% annual cut in the SRC's budget over the next four years, announced with the science vote last month, the outlook for science in the UK is looking slightly up. His cautious optimism is founded on the ABRC's decision to award the SRC greater shares than it could expect, in proportion to its annual expenditure, of the extra £4 million added to the science vote last October and of the £4.5 million to be spent on capital work in 1978/79.

A beneficiary of that optimism could be 'big science', long since neglected for research designated of national importance. "Nuclear physics, astronomy and space research have been battered" admits Professor Allen. For nuclear physics, in particular, he agrees that funding is now at the minimum viable level—almost. "We've nearly reached the baseline" he says, "which we must maintain to support a UK presence in nuclear physics".

And Professor Allen seems determined to have a say in the fate of British nuclear physics. Last December, he was at his own request appointed to the Council of the European Centre for Nuclear Research (CERN) in Geneva, now the major recipient of Britain's funds for high energy physics. He has already expressed his view that CERN's annual budget should be planned to remain at a plateau of about Sw Fr 560 million. The budget for 1978 is Sw Fr 615 million and many countries would like to see future budgets stabilise at about Sw Fr 600 million.

Not all British nuclear physicists, however, are in agreement with Professor Allen's baseline. Dr G. Stafford, Director of the UK Rutherford Laboratory thinks that a Sw Fr 560 million budget at CERN "will mean losing out on basic experiments". Such a low sum, he claims, "takes no regard for future development. By the 1980s existing machines will be old and at that level of funding we would not be able to replace them". On one point, though, Allen and Stafford do agree; that if future projections for the total UK nuclear physics budget are met, involving a 25% cut in spending over

the next five years, then there would be insufficient funds for a home-based programme and UK nuclear physics would be at an end. Stafford feels that the funding is at an absolute minimum.

The fate of high energy physics, however, is being pursued on another level. At the end of this week, Shirley Williams, Secretary of State for Education and Science, and a powerful lobbyist for the interests of scientists in the UK cabinet, will visit CERN.

The state in Britain of the other big science—astronomy and space science—is perhaps not quite so critical as that of high energy physics. Provided that there is no large cutback in funds over the next few years, radio astronomers should be able to maintain a competitive programme. Plans for a millimetre-wave radio telescope already have SRC approval: all that is now needed is Treasury approval for the £4.5 million it is likely to cost. And Jodrell Bank has plans to go ahead with extending its multi-telescope interferometer at an estimated cost of £2 million.

In astronomy it is the X-ray astronomers and space scientists who are showing most concern over their futures. Professor Ken Pounds of Leicester University claims that British X-ray astronomy is in a "fairly critical state". Its international reputation is higher than it has ever been but the prospects for the future are poor unless funding can be maintained at least at the present level. "If the UK is to get the best out of its subscription to the European Space Agency (ESA), then it must have a comparable home programme". But ESA can only fund one major new project in any particular discipline every two years, so each discipline is catered for approximately once every ten years. At the moment, for example, ESA has only one satellite for X-ray studies, EXOSAT. Therefore the UK must be able to bid for space on other satellites including NASA's Space Shuttle. This means maintaining a home based programme to the tune of £5-6 million.

Professor Allen's concern, however, is not entirely with the problems of 'big science'. He sees as one of his immediate priorities "making Sam's plans succeed" by continuing to give special attention to areas of national importance including microelectronics, marine technology and polymer engineering and making sure that schemes to improve collaboration between industry and universities are implemented.

Judy Redfearn

Skylab coming down

OFFICIALS at the US National Aeronautics and Space Administration are concerned that Skylab, last used in 1974 and still, at 85 tons, the largest manmade object in earth orbit, could fall to earth later this year.

Originally it was planned that Skylab would stay in orbit until 1980, when a manned space shuttle could attach a rocket motor to it that would send it into a higher orbit, or initiate a controlled re-entry.

Recent calculations, however, indicate that Skylab, which cost \$294 million to launch, could crash into the atmosphere later this year, and NASA officials are now working on a plan to command the still-operable steering rockets to send Skylab into a controlled tumble that would speed up re-entry.

The manoeuvre would be carefully calculated to bring the station back to earth on a steep descent over a broad area such as the Indian Ocean or the South Pacific. □

Letter from Sakharov and Meiman

IN October 1977, the US Association for Computing Machinery informed their Soviet opposite numbers that they would not cooperate with or sponsor international conferences in the Soviet Union until the Soviet attitude to human rights, exemplified by the case of Anatolii Shcharanskii, improved. Recently the ACM received the following message:

To the Association for Computing Machinery; to US scientists and engineers in computer technology

*Distinguished Gentlemen,
We'd like to express to you our deep gratitude for your resolute actions on behalf of Anatolii Shcharanskii. You have hit just the right nail. The Soviet authorities extremely appreciate the cooperation in science and technology, thus, there is nothing to induce them so factually and effectively as a refusal to maintain*

this cooperation.

We'd feel happy if your actions were followed suit by physicists on behalf of Professor Yuri Orlov and Andrei Tverdokhlebov, by biologists and medical specialists on behalf of Sergei Kovalev, as well as by writers and editors on behalf of Aleksandr Ginzburg.

Your courageous and noble stand is not simply ethically the best but the only practical one. Do not believe and do not take seriously any assertion that your decision allegedly could only embitter the Soviet authorities and aggravate the situation of Soviet scientists. Do not doubt that your human and professional solidarity will bring positive results.

*Nobel prize winner, Academician
A. Sakharov*

*Professor of Mathematics
N. Meiman*

CORN (maize) is the principal grain crop of the United States. Cash receipts from corn in the US in 1969 were nearly twice as much as for soybeans, and almost three times as much as for wheat. Peanuts (groundnuts) netted only 7% of the dollar value for corn. They have since become nationally prominent for presidential reasons. Peanuts, like soybeans, are legumes; they fix nitrogen and their seeds are good sources of proteins. Peanut protein is better balanced with respect to amino acids than is corn protein, but it is low in lysine, methionine and threonine.

Corn, in damp weather, and peanuts are good hosts for the growth of a mould, *Aspergillus flavus*, that produces aflatoxin, a potent carcinogen. The Food and Drug Administration (FDA) and the 'Delaney Clause' smiled benignly on aflatoxin, which is produced by God rather than the chemical industry, and is therefore not bannable. An 'action level' of 20 ppb of aflatoxin is used as a guideline for regulation. This level is carcinogenic in laboratory animals. In 1974, the FDA proposed to replace this level by a tolerance of 15 ppb for peanut products. Presumably this was intended to lower the cancer risk from aflatoxin somewhat, but the modest retreat is scarcely in accordance with the principle of total exclusion so sternly embodied in the Delaney Clause.

On 14 November, an FDA spokesman announced that 66 life-time cancers per 100,000 persons could develop from normal or expected ingestion of aflatoxin-contaminated pea-

nut and corn products. Presumably this figure would correspond to about one case per 100,000 persons per year. It was based on a combined estimated consumption of 2 ppb of aflatoxin from peanuts and 10 ppb from corn

Corn and Peanuts



THOMAS H. JUKES

products. This cancer rate may be compared with about 33 per 100,000 for lung cancer deaths, usually largely attributed to cigarette smoking, and about 168 for all US deaths from cancer. The estimate for aflatoxin corresponds to about 2,000 cancer cases for the entire US population.

I assessed the cancer hazard from diethylstilbestrol (DES) in beef production as not more than one case in 2,500 years in the entire US popula-

tion, based on pessimistic figures. This estimate, for some reason or other, was not challenged by the FDA lawyers who cross-examined me on 4 November at the hearings on DES in meat production. Perhaps this rather striking difference between hazards from DES and aflatoxin should be pondered by the FDA in setting priorities on bureaucratic regulations.

Aflatoxin should also be considered by vegetarians who tell us that we should eat corn, rather than feeding it to cattle, because we need to be less wasteful of food. The same school of thought advocates eating plant proteins, such as those of soybeans and peanuts, as replacements for beef and pork. However, when cattle and pigs eat corn and other feeds that contain injurious substances, they may filter them out, excrete them or metabolise them, especially if they are water-soluble. Being in a high position on the 'food chain' may have its advantages, even though we are frequently told that this is not the case.

The most active substances that inhibit mould growth belong to the antifungal group of the currently-criticised chemical pesticides. Also, oddly enough, the toxic effect of aflatoxin in rats can be reversed by dosing with DDT.

Two years ago an outbreak of hepatitis, with more than 100 deaths, was reported in India, associated with the consumption of corn that was contaminated with aflatoxin. Affected people could have consumed 2 to 6 mg daily for a month. About 1000 persons who recovered are being followed for liver cancer.

correspondence

GDR's state of science

SIR,—Under this presumptuous title your journal, which has built its reputation on a claim of fair and objective reporting, saw fit to publish the 'impressions' of an anonymous correspondent of his visits to unnamed biochemistry and microbiology laboratories in an undisclosed city of the GDR (13 October, page 548). The picture on the same page intimates that it might have been Leipzig. As a biochemist at the Karl-Marx University of Leipzig I feel that the readers of *Nature* deserve substantial factual information to do justice to the heading chosen by the editors.

The state of biochemistry is of course part of the general development of sciences in the GDR. In the last ten years alone funds for science have increased more than threefold and the percentage of the GNP going to science and technology has increased from 2 to 5. The life sciences have had their fair share in this development. As far as biochemistry is concerned it may suffice to mention that of the seven departments in medical faculties six are housed in new buildings (Rostock, Berlin, Leipzig, Halle, Jena, Magdeburg). In the Academy of Sciences there have been founded eight institutes of life sciences with a sizeable representation of biochemistry, in particular in the Institut für Molekularbiologie, Berlin-Buch, and the Institut für Biochemie de Pflanzen, Halle. All establishments are headed by prominent scientists rather than by administrators. The development of research in the life sciences is largely based on prognostic documents, worked out by some hundreds of scientists themselves, which have been accepted by government as a framework for implementation.

The vast majority of scientists in the GDR go along with the tenets held by most thoughtful scientists elsewhere—that freedom of research is tied indissolubly to social responsibility. All scientists loyal to the GDR have equal chances regardless of creed and there is certainly no pressure to join the Socialist Unity Party or any other. As far as the spirit of young biochemists is concerned all I have experienced is enthusiasm rather than despondency. There is a general feeling of social security and optimism shared by the scientists with all citizens of the GDR which is based on the record of uninterrupted progress of our socialist society which does not know economic

crises or unemployment. There is of course no denying that we wish to have more foreign exchange at our disposal for more equipment, literature and travels. But we know that this depends in the first line on the efforts of our workers to increase export.

As far as conditions at the Karl-Marx University are concerned, scintillation counters, analytical and preparative ultracentrifuges from internationally renowned manufacturers as well as the internationally leading biochemical journals are in fact available. The Karl-Marx University maintains an effective cooperation with British universities and biochemists.

For many years there has been a regular exchange of staff and students, biochemists included, between the Karl-Marx University and the universities of Leeds, Manchester and York. Two biochemists of the Karl-Marx University, one of them myself, are members of The Biochemical Society of Great Britain.

The growth of biochemistry is reflected in the number of members of the Biochemische Gesellschaft der DDR, whose membership has doubled over the past few years and now totals 610. The scientific life is an active one and a multitude of national and international meetings are held including the Federation of European Biochemical Societies (FEBS) Advanced Courses. It is to be hoped that a good many readers of *Nature* will participate at the 12th FEBS Meeting to be held in Dresden, 2–8 July, 1978 and will be able to judge for themselves both the general state of life and of science in the GDR.

E. HOFFMAN

Karl-Marx Universität, Leipzig, GDR

Discarding the diet-heart hypothesis

SIR,—No, Mr Rivers (3 November, page 2), I am not a heretic, I am a scientist. Science is for me a religion both because it opens an endless wonderland and because it works. It is, in a needy world, a way to solve problems, as are Buddhism and Christianity and Judaism and the other religions, each in their own way. The way science works is quite simple but not widely understood. Using the best available evidence the scientist formulates an hypothesis and then he and his peers set out to disprove that hypothesis. We never prove in science, we advance to new understanding by

disproofs. The hazard, as Platt has so eloquently said in his essay 'The step to man', is that scientists may become so enamoured with some favourite hypothesis that they never test it. Or they become intrigued with the testing hardware and dawdle or they refuse to accept the disproving results of good tests. The last has happened with the diet/heart hypothesis. A noisy clutch of scientists is defending, excusing, delaying, procrastinating over the decision to discard a disproven hypothesis. Their tactics prevent progress.

Sir Andrew Huxley was lately quoted (8 September, page 95) as saying that the most sinister behaviour for scientists is to refuse to test an hypothesis (say the inheritance of intellectual ability) because it would be thought socially or politically wicked to raise a question whose answer might disagree with some preconception. No less sinister is the tactic of the diet/heart enthusiasts who, by their noisy propaganda hope to coddle and prolong the diet/heart hypothesis. That hypothesis has been shown wrong by scientific method. I believe that because I believe in the scientific method.

The heretics, Mr Rivers, are selling corn oil.

GEORGE V. MANN

Nashville, Tennessee

Research and culture

SIR,—It is sad, when it comes to defending support for basic science, that *Nature* editorialists should resort to stating the obvious, that is that basic research is a cultural activity ('How much further can the pendulum swing?' 27 October, page 743).

Sad but not surprising, considering how many educated persons, including scientists, seem to ignore this fact or are confused by the distinction between civilisation and progress. Civilisation is culture. Encouraging cultural activity is maybe the best investment for the future of a nation; it is an investment in enlightenment, a non-depreciating quality (whether or not research comes up with the cure for cancer—that would be progress).

After talking with friends in the British theatre, however, who have been out of work for a while now, it might not be such a good idea, after all to remind the government that research is a cultural activity.

GIUSEPPE ZACCAI
NORINE ZACCAI

Domène, France

news and views

OUR picture of the organisation of genes in higher organisms has recently undergone a revolution. Analyses of eukaryotic genes in many laboratories¹⁻¹⁰, studies of globin, ovalbumin, immunoglobulin, SV40 and polyoma, suggest that in general the coding sequences on DNA, the regions that will ultimately be translated into amino acid sequence, are not continuous but are interrupted by 'silent' DNA. Even for genes with no protein product such as the tRNA genes of yeast and the rRNA genes in *Drosophila*, and also for viral messages from adenovirus, Rous sarcoma virus and murine leukaemia virus, the primary RNA transcript contains internal regions that are excised during maturation, the final tRNA or messenger being a spliced product.

The notion of the cistron, the genetic unit of function that one thought corresponded to a polypeptide chain, now must be replaced by that of a transcription unit containing regions which will be lost from the mature messenger—which I suggest we call introns (for intragenic regions)—alternating with regions which will be expressed—exons. The gene is a mosaic: expressed sequences held in a matrix of silent DNA, an intronic matrix. The introns seen so far range from 10 to 10,000 bases in length; I expect the amount of DNA in introns will turn out to be five to ten times the amount in exons.

This model immediately accommodates two aspects of the genetic structure of higher cells. Heterogeneous nuclear RNA clearly is the long transcription products out of which the much smaller ultimate messengers for expressed polypeptide sequences are spliced. The unexpected extra DNA in higher cells, the excess of DNA over that needed to code for the number of products defined genetically, now is ascribed to the introns.

What are the benefits of this intronic/exonic structure for genes? For the sake of argument let us assume that the splicing mechanism is general and independent of the specific gene or the state of the cell, reflecting simply some secondary structure in the RNA. For example, base-pairing in the messenger could generate sites which would serve as signals for enzymes, such as those that excise tRNAs from their precursors, to cut out a section. The cut would be

Why genes in pieces?

from Walter Gilbert

resealed by an RNA ligase. Even if RNA processing is general, the presence of infilling sequences can speed evolutionary

Single base changes, the elementary mutational events, not only can change protein sequences by the alteration of single amino acids but now, if they occur at the boundaries of the regions to be spliced out, can change the splicing pattern, resulting in the deletion or addition of whole sequences of amino acids. During the course of evolution relatively rare single mutations can generate novel proteins much more rapidly than would be possible if no splicing occurred.

Furthermore, the splicing need not be a hundred per cent efficient; changes in sequence can alter the process so that base pairing and splicing occurs only some of the time. Even mutations in silent third base positions, could modify the joining so that the products of a single transcription unit can be both the original gene product and a new product, also synthesised at a high rate. Evolution can seek new solutions without destroying the old. A classic problem is resolved: the genetic material does not have to duplicate to provide a second copy of an essential gene in order to mutate to a new function. Rather than a special duplication, the extra material is scattered in the genome, to be called into action at any time. After a new gene function appears, if a higher level of product is needed, there will be selective pressure for gene duplication (as well as pressure for the loss of the introns in highly repeated genes). One consequence of the intronic model is that the dogma of one gene, one polypeptide chain disappears.

A gene, a contiguous region of DNA, now corresponds to one transcription unit, but that transcription unit can correspond to many polypeptide chains, of related or differing functions.

Recombination now becomes more rapid. Since the gene is spread out over a larger region of DNA, recombination, which should be hampered in higher cells by the inability of DNA molecules to get together, will be enhanced. Furthermore, if exonic regions correspond to functions put together by splicing to form special combinations in the finished protein, then recombination within introns will assort these functions independently. Middle repetitive sequences within introns may create hot spots for recombination to rearrange the exonic sequences.

Recombination within introns will generate curious genetic structures for eukaryotic genes. Structural mutations should be clustered, separated by long distances from mutations in other exons. Mutations in different functions may be interspersed, when one product's intron becomes another's exon.

According to this view, introns are both frozen remnants of history and as the sites of future evolution. Nevertheless, they could also have other roles. Specific recombinations between introns can bring together exons into a transcription unit to make special differentiation products. Specific new splicing patterns could be turned on by special gene products. A differentiation pathway may be determined by the appearance of a new splicing enzyme, calling forth new proteins out of the heterogeneous nuclear RNA.

On this can be based a striking hypothesis to explain the behaviour of immunoglobulin heavy chains. At an early stage of the immune response a single lymphocyte can synthesise two different immunoglobulins, IgM and IgD, with the same idiotype; two different constant portions attached to the same V_H region. This may be the result of a V_H region translocating by recombination within an intron near the constant genes so that a transcription unit is formed for a $V_H-C_H-C_\delta$ message. Splicing can then create contiguous messenger sequences for V_HC_H and V_HC_δ chains. The switch from IgM to IgG might be a new translocation of the V_H gene, but, alternatively, it may be a new enzyme that changes the processing of a $V_H-C_{H\gamma}C_\delta-C_\gamma$ message to produce a V_HC_γ product. □

Walter Gilbert is American Cancer Society Professor of Molecular Biology at Harvard University.

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Restriction endonucleases: a new role *in vivo*?

from Richard J. Roberts

FEW enzymes have been exploited as thoroughly as the bacterial restriction enzymes. In recent years they have been instrumental in dramatic advances in DNA sequence analysis, genetic engineering, and studies of gene structure and function. Yet surprisingly little is known of their biological role. It has become almost axiomatic to associate them with a defence mechanism by which bacteria insulate themselves against invasion by unwelcome foreign DNA. This view has its origins in the genetic phenomenon of host-controlled restriction and modification. Two enzymes mediate this process: an endodeoxyribonuclease (restriction enzyme) which degrades DNA, and a methylase (modification enzyme) which protects DNA against the action of the restriction enzyme. Because both enzymes coexist in the same cell, the cell's own DNA is protected by the action of the methylase, whereas incoming foreign DNA, which lacks the proper modification, is susceptible to destruction by the restriction enzyme.

Two different types of restriction enzymes have been found and can be distinguished by their mode of cleavage. Although both recognise a specific sequence within a DNA molecule, the Type I enzymes cleave at random sites remote from that sequence and give heterogeneous products, while the Type II enzymes cleave at specific sites within or close to the recognition sequence. It is these latter enzymes that have enjoyed such popularity as the molecular scalpels of the biochemist. Perhaps the best-known example is *EcoRI* from *Escherichia coli* which recognises the sequence, G⁺AATTC, and cleaves at the site indicated by the arrow. It produces specific fragments which carry short, single-stranded extensions at each end allowing them to reanneal and become a substrate for a DNA ligase. Thus, any two *EcoRI* fragments can be joined, which has made possible the powerful techniques of genetic engineering.

But paradoxically, the very enzymes which are thought to prevent the exchange of DNA *in vivo* are precisely those which facilitate that exchange *in vitro*. Could it be that the restriction enzymes have a dual role *in vivo* and catalyse both degradation and synthesis just as they do *in vitro*? This possibility has been raised previously but until now it had not received serious attention. It seemed unlikely that bacteria could be as smart as molecular biologists, but apparently they are. For recent

experiments by Chang and Cohen (*Proc. natn. Acad. Sci. U.S.A.* **74**, 4811; 1977) show that an *E. coli* strain carrying the *EcoRI* restriction enzyme can indeed mediate genetic engineering *in vivo*. In a series of elegant experiments, they have demonstrated that a variety of site-specific recombination events can be catalysed *in vivo*.

Their experiments centred around the fact that a chloramphenicol (Cm) resistance gene contains an *EcoRI* site located so that cleavage will inactivate the gene. Restoration of gene activity requires precise rejoining at the *EcoRI* site. It was first shown that such precise rejoining could be accomplished *in vivo* by transfecting *E. coli* with two *EcoRI* fragments, each containing a part of the Cm resistance gene. Chloramphenicol-resistant transformants were recovered. Then a plasmid, pSC352, was constructed in which the same Cm gene was inactivated by the insertion of an *EcoRI* fragment. Transfection of pSC352 into an *E. coli* strain (C600, pMB4) which contained the *EcoRI* restriction enzyme resulted in a few transformants (6×10^{-5} of all transformants) which had acquired Cm resistance. *In vitro* analysis showed that such transformants arose from precise excision of the inserted *EcoRI* fragment. In more extensive experiments, they showed that *EcoRI* promoted site-specific recombination could involve multiple and physically separate fragments of plasmid DNA. In each case, the plasmids used for transformation were prepared so as to be unmodified against the action of the *EcoRI* restriction enzyme. What if the sites are already modified? To answer this question, Chang and Cohen propagated pSC352 in *E. coli* C600 (pMB4), where it is modified by the *EcoRI* methylase, and then observed the frequency of excision of the inserted fragment under normal growth conditions. Chloramphenicol-resistant clones developed with a frequency of about 10^{-9} . In this case, therefore, even the presence of the correct modification enzyme within the cell was insufficient to prevent site-specific recombination.

Although the observed frequency of these events *in vivo* is extremely low, their importance should not be underestimated. They raise anew the question of the biological role of restriction enzymes, and have implications for the current heated controversy about *in vitro* recombinant DNA experiments. Because these observations were made in the laboratory, it is difficult to assess their relevance to processes which might occur in the natural environment. Nevertheless, even if they occur at the low frequency observed by Chang and Cohen, the

magnitude of the bacterial population on the face of this Earth would still mean that the phenomenon could be of considerable importance to bacterial evolution. Such a possibility has been raised previously (Reanney *Bact. Rev.* **40**, 552; 1976). Clearly, it is necessary that experiments be carried out under conditions resembling those *in vivo* for if indeed bacteria routinely exchange genetic information in this way, such exchange would not be limited to DNA originating from prokaryotes. We would be forced to the conclusion that species barriers exist, not because of a physical barrier to the exchange of genetic information, but rather because a functional barrier prevails. If such is the case, it would surely provide the strongest argument against the strict regulation of recombinant DNA experiments *in vitro*.

This question of frequency has other profound implications. If the frequency can be raised in the laboratory, then it may be possible to construct recombinant DNAs in this way. Shotgun experiments might merely require transformation of the appropriate strain with intact DNA. In this way, both the biochemistry and the present regulations could be circumvented. Of course, ethical questions remain and, presumably, the next round of guidelines for recombinant DNA research will take account of these new observations. It will be of some interest to see how they are phrased. Perhaps all experiments involving recombination, whether *in vivo* or *in vitro*, will fall under their jurisdiction; this could include the whole of classical genetics. Even the most ardent bureaucrats will find it impossible to regulate recombination in the environment. Plants, insects, animals, do it all the time. The line may have to be drawn in the laboratory. There sex will require a Memorandum of Understanding and Agreement, while elsewhere it remains unrestrained. □



A hundred years ago

THE New York *Tribune* gives an account of a public exhibition in that city of Edison's Phonograph, which seems to have been very successful. The tones reproduced by the vibrating disk of the machine were so distinct that they could be heard and understood in different portions of the crowded room.

From *Nature* **17**, Feb. 7, 291; 1878.

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A RELIABLE method of assessing the mutagenicity of chemical and physical agents in mammalian cells would be invaluable. Visible chromosome damage such as breaks, and translocations has been used, but has proved rather disappointing so far; it is not as sensitive as could be wished, and while it can be effective for heavy doses of some mutagens, such as X rays, it often yields unsatisfactory results in terms of normal dose rates of ingested substances (as seen for instance in the confused literature on chromosomal breakage and LSD). Perry and Evans (*Nature* **258**, 121; 1975) showed that the number of sister chromatid exchanges (SCEs) per cell is a far more sensitive indicator of exposure to both physical and chemical mutagens than is the number of visible chromosomal aberrations, but it has not yet been proven that the presence of increased numbers of SCEs necessarily indicates actual damage to the genetic information of the cell. But now Carrano *et al.* (page 551 of this issue) have provided stronger evidence that the number of SCEs seen in cells after treatment with a range of known mutagens may be directly related to genetic damage and mutation rate, in which case it could represent a sensitive and easily quantified test of mutagenicity.

Sister chromatid exchange can be seen in chromosomes studied at the metaphase stage of cell division. At this stage each chromosome is made up of two equal chromatids which arise when the chromosomal DNA is replicated at the synthetic (S) phase of the cell cycle, a few hours before cell division starts. If material has been exchanged between these two sister chromatids by the metaphase stage it means that DNA breakage and repair must have

Testing for mutagenicity

from E. H. R. Ford

taken place between S phase and metaphase. Precise interchange of material need not of itself alter the genetic information of a cell, so the significance of visible SCE as a measure of possible genetic damage or mutation has not so far been clear.

Although SCE was described by J. H. Taylor *et al.* 20 years ago (*Proc. natn Acad. Sci. U.S.A.* **43**, 122; 1957), using an autoradiographic technique, it is only since S. A. Latt (*Proc. natn Acad. Sci. U.S.A.* **70**, 3395; 1973) introduced an optical method for differentiating sister chromatids that SCE has readily been studied. A newly replicated chromatid which has incorporated the thymidine analogue BUDR can be differentiated from its older sister by fluorescent, Giemsa staining or immunological techniques (since the BUDR quenches the fluorescent or staining property of the chromatin).

The baseline SCE frequency in untreated human lymphocytes is 5–15 per cell (Latt & Juergens in *Population Cytogenetics* (Eds Hook, E. B. & Porter, I. H., Academic Press, 237; 1977). Alkylating agents such as ethylmethanesulphonate, mitomycin C or nitrogen mustard (which can all also induce visible chromosomal damage) considerably increase the number of SCEs per cell at concentrations where no other visible morphological damage is caused to chromosomes, indicating that SCE number is a sensitive test for chromosome damage.

Carrano *et al.* have now shown that

in Chinese hamster ovary cells there is a strong and linear relationship between the induction of SCEs and of mutations producing resistance to 8-azaguanine (mutations mainly at a particular locus, the hypoxanthine phosphoribosyl transferase (HPRT) locus.) They used four mutagenic agents; the alkylating agents, ethylmethanesulphonate (EMS) and N-ethyl-N-nitrosourea (ENU), mitomycin C (MMC) which is also a cross-linking agent, and proflavine sulphate (PRO), which intercalates into DNA. Each agent produced increases in the SCE and mutation rates, and for each there was a linear relationship between SCE and mutation rate—but this was different for each agent. ENU was the least efficient inducer of SCEs compared with mutations, and MMC the most efficient. The authors have been able to calculate the number of mutations per cell which correspond to one SCE, ranging from 0.08 for MMC to 1.2 for ENU.

If certain extrapolations can be made, namely that the marker used gives a representative mutation rate comparable to that at other loci, that human cells behave similarly to those of the Chinese hamster and that the SCE/mutation ratio *in vitro* can be extrapolated to cells *in vivo*, it would be possible to estimate the mutagenicity of various physical and chemical agents simply by counting the number of SCEs found in human lymphocytes.

Carrano *et al.*'s findings need to be confirmed and the validity of these extrapolations determined. But if Carrano *et al.*'s suggestions are confirmed we may soon have a greatly improved, simple and direct method of assessing the mutagenicity of all sorts of agents.

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Preplanetary disk?

from Andrew Fabian

THE formation of stars is shrouded by dust. The massive gas clouds within which they form by gravitational collapse are cold and seeded with dust grains. Optical radiation from the newborn star is scattered and absorbed by these dust clouds, then reradiated at infrared wavelengths. Consequently it is often easier to deduce more about the properties of the gas and dust than the star. This means that little is known observationally about this important phase of a star's life.

From a theoretical point of view the situation is somewhat happier, at least until the observations improve. The collapse and fragmentation of a cold gas cloud under gravity has been discussed for decades. Problems emerge,

not the least of which are to do with the rotation and magnetisation of the star. Perfectly spherically symmetrical collapse seems most unlikely, especially when it is recalled that stars are observed to rotate at speeds up to and approaching breakup speed. Many stars are, moreover, members of binary (or multiple) pairs in orbit about each other. A collapsing rotating gas cloud is likely to flatten out and form a disk with a bulge in the centre—not too dissimilar in shape to our Galaxy. The bulge will form the stars and the disk what? Planets perhaps?—A tempting possibility considering the disk-like concentration of the major and many of the minor bodies of the Solar System. Gravitational collapse is halted within the central star, at least for the time being, by the onset of nuclear fusion, but accretion will continue within the disk until it is dispersed. Turbulent or magnetic viscosity in a disk as dense as might be associated with a newly

forming star causes matter to spiral in at the expense of angular momentum, which flows outward. We have our friend the accretion disk, which has been much discussed in the context of accretion onto black holes, both in X-ray binaries and galactic nuclei. A start was made on modelling accretion disks around newly formed stars a few years ago by D. Lynden-Bell and J. Pringle (*Mon. Not. Roy. Astr. Soc.* **168**, 603; 1974). Clearly, observational evidence of such a disk around a newly formed star is of great interest.

R. I. Thompson, P. A. Strittmatter, E. F. Erickson, F. C. Witteborn and D. W. Strecker have made a good start (*Astrophys. J.* **218**, 170; 1977) by combining their own infrared spectra of some highly reddened emission-line objects with unpublished optical continuum data of one of the objects taken by S. Grandi. MWC 349 and LK H α 107 are objects from the Mount Wilson, and Lick, catalogues of emis-

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sion-line objects. Both objects appear to be newly formed stars embedded in dense gas and dust clouds. The ultraviolet radiation of such bright young stars rapidly photoionises the surrounding gas and is thereby completely absorbed. Recombination within the resulting HII region is then responsible for the bulk of the observed emission lines. Reversing this reasoning, we see that a study of the strengths of such lines ought then to be a monitor of the ultraviolet flux of the star. Such a technique has been employed for some time, but in the case of these highly obscured objects, the emission lines must be dereddened before any reliable estimate may be made.

This is where the combination of aircraft and ground-based infrared spectroscopy comes in, for the Paschen and Brackett lines of hydrogen are much less affected by the dust reddening than the optical Balmer lines. A comparison of observed line ratios with those predicted on the basis of recombination within an HII region allows an extinction curve to be obtained. The authors use the strength of Brackett γ to estimate the emission measure of the HII region and thence (with a distance estimate) the spectral type and properties of the central star. In the case of MWC 349, a 30 solar mass 06.5 star is implied. Whilst other emission lines and radio data are consistent with this result, the observed visual magnitude is not. The inferred star is about 11 times too faint to account for the observations when reddening (using the enormous dereddening factors deduced above) is included. This might be due to a mistake in the reddening corrections, but there are problems, too, with the known 40-year brightness decrease of this object, and the flatness of its optical continuum, both of which are uncharacteristic of isolated stars.

The authors thus invoke another component—a preplanetary disk which is accreting onto the central star. It is easy to show that the long wavelength spectrum of such a disk is flatter than that of a star if both radiate as blackbodies. The inner regions of the disk are hottest, but have a smaller area than the cooler outer regions and the balance gives a spectral slope of 1/3, in agreement with the optical data. At ultraviolet wavelengths the stellar radiation dominates the ionisation of the HII region, but the disk is assumed dominant in the optical. An accretion rate is thereby deduced which together with the 40-year variability timescale suggests a disk mass at least 1% that of the Sun.

Whilst the evidence does not decisively indicate an accretion disk, or demonstrate that planets would or could form in such a disk, it does seem that the authors are justified in claim-

ing that "MWC 349 is certainly by far the best now known candidate for a

newly formed star with a surrounding preplanetary disk." □

Nations and numbers 1977

from Robert M. May

EACH year, the Environmental Fund (1302 Eighteenth St, Washington, DC) pulls together UN and other estimates, to produce a large chart of population data for individual countries, and for regional groupings. In addition to population magnitudes, and birth, death and net growth rates, the chart gives a changing selection of other statistical information pertaining to aspects of the human condition. In 1975 this included country-by-country patterns of age structure and urbanisation (see *News and Views* 261, 12; 1976), and in 1976 the number of acres of arable land per person, and the past and present figures for import or export of cereal grain (*News and Views* 265, 101; 1977).

All these numbers are uncertain. In the past, I have indicated some of the reasons, for countries as different as the USA and China. There is a major discontinuity between the Environmental Fund's world figures for 1976 and for 1977, which stems from a revision in the Indian population data. Previous numbers had been based on the 1974 population, as estimated by the International Statistical Program Center (ISPC) of the US Bureau of the Census; increasing this by the reported population growth rate gives around 657 million Indians in 1977. ISPC and the UN now base their estimates on the 1973 Indian census, giving a new figure of 642 million in 1977. This at a stroke erases 15 million bodies from the estimated world population. As the Environmental Fund notes, it also "indicates that either one or two Indian censuses were grossly in error or that Indian vital statistics are vastly worse than had been generally supposed". In eight other countries (Guinea, Ivory Coast, Senegal, Madagascar, Cameroon,

Lesotho, Saudi Arabia, United Arab Emirates) the results of new national censuses indicate population sizes greater than had been thought.

Overall, the world population in mid-1977 was around 4,307 million, growing at an average rate of 2.1% per annum. Highest growth rate was in Libya, with 4.1% per annum; Kuwait's 6.1% was disqualified, as influenced by immigration. Lowest was Portugal, with -0.4%. Many of the local differences in average population growth rates (for example 3.6% in Gaza versus 2.1% in Israel) have political consequences.

Regional patterns over the sweep of the past 27 years are interesting. Since mid-1950, the world population has grown from around 2,543 to 4,307 million, representing an average growth rate of 2.0% per annum over that period (as contrasted to the 1977 figure of 2.1%). The regional components are as set out in the table.

Another pattern documented in the 1977 Environmental Fund chart concerns literacy rates. These rates for individual countries have been collected from various sources (UNESCO, US Department of State, CIA and others), and they are likely to be vagariously defined and of questionable accuracy. Even so, it is interesting to plot the literacy rates against the population growth rates for the 157 countries included in the chart. This is done in Fig. 1. The black squares are regional averages (Africa, 22% literacy and 2.8% population growth rate; Asia, 49 and 2.4; North America, 99 and 0.8; Latin America, 65 and 2.8; Europe, 96 and 0.6; USSR, 98 and 1.0; Oceania, 84 and 1.8), and the large star is the global average (59% literacy and 2.1% growth rate).

No simple message emerges from Fig. 1. It does suggest that low population growth (below 1% say) is correlated with high literacy, but even here Portugal

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Region	Population in mid-1950 (millions)	Population in mid-1977 (millions)	Average growth rate (% p.a.)	1977 growth rate (% p.a.)
Africa	219	451	2.7	2.8
Asia	1,409	2,506	2.1	2.4
North America	166	247	1.5	0.8
Latin America	164	342	2.7	2.8
Europe	392	479	0.7	0.6
USSR	180	259	1.3	1.0
Oceania	12	22	2.2	1.8

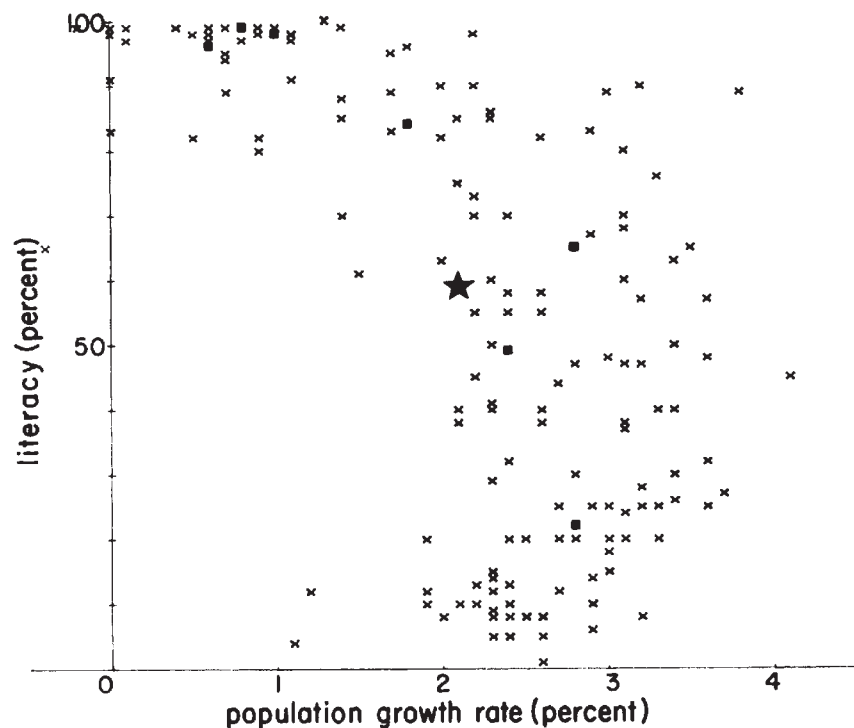


Fig. 1 Percentage literacy is plotted against annual population growth rate for 157 countries (a few of the crosses represent two countries). The solid squares are regional averages, as described in the text; the large star is the global average.

(-0.4% population growth with 65% literacy) is an exception. At the other end, although there are a lot of countries with population growth rates in the range

2-4% and literacy rates below 30%, the figure does not support any unambiguous correlation between high population growth and low literacy. □

New ways to look at X inactivation

from M. Bobrow

MAMMALIAN embryogenesis is very sensitive to alterations in the 'balance' of genetic material. Additions or losses of whole chromosomes usually produce severe phenotypic derangements. In this context, the sex chromosomes are somewhat of an anomaly. The Y chromosome contains little if any genetic information other than that directly relevant to inducing testicular differentiation of the primitive gonad. The X chromosome is large and contains at least several hundred gene loci having nothing to do with sex differentiation. However, the female develops normally with two X chromosomes, while the male manages at least as well on only one.

The crucial mechanism behind this phenomenon is a regulatory system which allows only one X chromosome to remain active in a diploid cell. First proposed in 1961 (Lyon *Nature* 190, 372) the 'Lyon hypothesis' has been proven

substantively correct, but details of the process are still obscure (well reviewed by Gartler & Andina *Adv. hum. Genet.* 7, 99; 1976).

The basic hypothesis states that, at some stage during early embryogenesis, all X chromosomes bar one are inactivated and are no longer available for transcription. Which X remains active in any given cell is randomly determined. The process involves a whole chromosome rather than individual genes, and is accompanied by physical changes in the chromosome—it is visible as a highly condensed body during interphase (the Barr body) and the timing of its DNA synthesis is shifted to a later stage of the cell cycle than its active homologue. Whatever the nature of the inactivation process it is heritable, so that descendants of a given cell form a clone. Every normal female is thus a somatic cell mosaic, some cells having an active paternally-derived X, and others an active maternal X. The growth of knowledge concerning biochemical markers of X chromosome activity, and the powerful new techniques for manipulating the genetic constitution

of mouse embryos (by forming chimaeras, for example), has led to a recent wave of interest in the timing, extent and randomness of X inactivation.

Cytological evidence of X inactivation first appears in the early blastula of the mouse (± 40 cell stage). Functional inactivation was studied in a most elegant experiment: single cells from 3.5-4.5 d blastocysts were injected into host blastocysts, and formed chimaeric mice displaying patches of activity of both X chromosomes of the injected cell—implying that cell transfer had preceded X inactivation. (Gardner & Lyon *Nature* 231, 385; 1971) Studies of 'patch size' of X-linked markers in adult female heterozygotes lead to rough estimates of 10-60 cells present in the embryo at the time of X inactivation.

Another approach to the timing of X inactivation is the biochemical analysis of X-linked gene products in the early embryo. Before inactivation, XX embryos should produce twice as much gene product as their brothers, which would lead to a bimodal distribution of gene activity. An obvious pitfall is that unimodal distributions of enzyme activity in very early embryos may reflect pre-fertilisation oocyte transcription.

A recent example of this approach (*Nature* 270, 599; 1977) is the study of Monk and Kathuria, who analysed HGPRT activity in single mouse embryos. They found a unimodal distribution of activity in 8-cell embryos and in pre-implantation blastocysts—at which stage the embryonic genome is certainly active. There is evidence of embryonic synthesis of HGPRT from a very early stage of embryogenesis, and this finding would therefore suggest that X inactivation has occurred well before implantation; earlier than suggested by the other approaches. This discrepancy could be due to methodological difficulties in one or more of the techniques; inactivation occurring at different times in different embryonic cells; or to inactivation not affecting the whole chromosome simultaneously. An intriguing speculation would be that regions of the chromosome are inactivated as their transcriptional products are required in the differentiating embryo.

The concept of random inactivation seems to be generally valid, but three major exceptions have been discovered. X/autosome translocations and structurally abnormal X chromosomes in both man and mouse show preferential inactivation of one or other X chromosome—but these situations have only been studied in adult animals and may represent post-inactivation cell selection. Information on inactivation in early embryos with X/autosome translocations would be most interesting. In marsupials, the paternal X chromosome is preferentially inactivated and a similar situation pertains in the extra-embryonic membranes of rodents (refs in Gartler &

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Andina *op. cit.*). This seems to imply some form of chromosome 'imprinting' to mark the paternal X chromosome, and it has been suggested that this is a fundamental and primitive form of X inactivation, from which some cells of the eutherian embryo have learned to escape. That imprinting is not a necessary prerequisite of X inactivation has been finally demonstrated by Kaufman, Guc-Cubriilo and Lyon (this issue of *Nature*, page 547) who have provided cytological evidence of X inactivation in the absence of any paternal genetic contribution, using parthenogenetic embryos created by suppression of polar body formation.

Despite a variety of interesting suggestions on mechanisms for permanently modifying DNA bases or structural configuration (see refs in Gartler &

Andina *op. cit.*) virtually nothing is known of the biochemical or biophysical basis of X inactivation. This, and many other aspects of this fundamental process of mammalian genetic regulation, may become amenable to investigation following the exciting discovery of Martin *et al.* (*Nature*, 271, 329; 1978). These workers have demonstrated that mouse teratocarcinoma cell lines maintained *in vitro* show biochemical evidence of X inactivation if they are grown under conditions which allow them to initiate morphological differentiation. If this experimental model turns out to be as useful as it promises on first sight, our understanding of the process of X inactivation, and of whole-chromosome genetic regulatory mechanisms, may advance rapidly over the next few years.

□

Smoothing primaeval chaos

from Paul Davies

THERE are two conflicting philosophies about the nature of the Universe. The first, which meshes well with traditional religious beliefs, is that the world we live in is a very special sort of place, elaborately constructed to be just the way it is and no other. The second holds that there is nothing really remarkable about the Universe, given the laws of physics. According to the latter, the world is a pretty random sort of place. The nexus of this controversy focuses on the creation event—the so-called big bang—which apparently took place about 15 billion years ago and represents the origin of all space, time and matter. Assuming the reality of this universal genesis, the question naturally arises as to the precise condition in which the Universe was created. Some cosmologists contend that it began in a highly special and extremely improbable condition, others that it started out as though God had chosen the world randomly from all possible alternatives.

To defend the second philosophy, it is necessary to demonstrate that there exists a very wide range of initial conditions that would cause the Universe to subsequently evolve into something closely similar to what we observe today. Another way of expressing this is to say that our basic world structure is stable against random changes in its original state: that almost any sort of big bang would have led inevitably to a Universe of the type we have.

In the past few years a lively debate

has arisen around this question, as our understanding of the primaeval cosmos improves, both through astronomical discovery and theoretical modelling. The central observational fact is that, on an extragalactic scale, the Universe appears to be very uniform and regular, both as regards the distribution of matter in space and the systematic pattern of galactic recession which reveals the overall expansion of the cosmos. The great mystery is: why is the Universe so orderly? Was it simply created in this remarkably uniform condition, or has it contrived to evolve order out of primaeval chaos? A further twist to the problem is that without some degree of irregularity the galaxies themselves would not have formed. Galaxies arose because certain regions of the primaeval gases erupting from the big bang were slightly denser than others, and the locally enhanced gravity associated with this clumping proceeded to trap the surrounding material and prevent it from dispersing under the action of the universal cosmological expansion. In this way, initially rather tenuous irregularities inexorably grew in size and contrast until they became the isolated entities that we now observe.

Several years ago intensive investigations began into the fate of a cosmos which starts out with a large amount of turbulence. Several mechanisms exist which would operate to smooth out the primaeval turbulence. For example, fluid viscosity can damp away chaotic motions and restore orderly flow in many everyday systems. In a cosmological context, the viscosity of

neutrinos (elusive subatomic particles) was suggested by the American cosmologist Charles Misner as a cause of viscous damping operating at about 1 second after the big bang, when the Universe had a temperature of 10 billion degrees. Other damping processes have since been discussed; quantum particle production around 10^{-23} s, graviton collisions at 10^{-20} s, hadron collisions at 10^{-6} s, microscopic black hole production, and so on. All these processes operate to reduce the turbulent chaos and establish a more orderly and regular arrangement of matter.

On the face of it, one could well suppose that, through these many mechanisms, even a highly irregular primaeval cosmos would soon convert into something like the present condition. The snag is that, according to the second law of thermodynamics, the establishment of order out of chaos has to be paid for by an increase in total entropy, most of which appears as heat energy. The heat of the primaeval Universe can still be detected as the celebrated cosmic background thermal radiation, which provides us with a measure of the entropy of the Universe. Although the temperature of this radiation is now very low (about 3 K), it still represents a vast amount of entropy per atom of matter.

In a recent paper (*Mon. Not. R. Astr. Soc.* 181, 719; 1977) John Barrow of Oxford University and Richard Matzner of the University of Texas at Austin have re-examined the relationship between the cosmic entropy and the primaeval turbulence. They point out that although the entropy is large (indicating that a definite amount of dissipation occurred at some stage in the past) it is obviously still finite, and can be used to provide an upper limit on the amount of primaeval disorder. They argue as follows: observations of the thermal background radiation and studies of nuclear processes in the primaeval matter indicate that the Universe was highly isotropic (regular in all directions) as early as a few minutes after the creation event, so any initial anisotropy must have been dissipated before that time, and converted into heat radiation. Now the energy of heat radiation is greater at earlier moments, as one probes closer to the initial creation event, because the Universe is more compressed and so the temperature is higher. Similarly, the energy of the anisotropy turbulence also grows as one approaches the beginning. What Barrow and Matzner point out is that the anisotropy energy grows much more rapidly than the heat energy. The crucial consequence of this observation is that the earlier the anisotropy was dissipated, the

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greater the heat (that is, entropy) it would have produced. For example, if dissipation occurred as early as the so-called Planck era (10^{-43} s) when quantum gravity effects were important, then even a minute amount of initial anisotropy would have produced too much entropy.

Without a detailed understanding of the dissipative mechanisms in the very early stages, it is impossible to place precise numerical constraints on the permitted degree of primaeval turbulence, but the authors conclude on general grounds that an initially highly chaotic cosmology can be ruled out. There is still the problem of accounting for the existence of galaxies in this quieter scenario, a phenomenon about which only rudimentary understanding has been gained.

If Barrow and Matzner's ideas continue to hold for more elaborate models of the big bang, it seems that we shall inevitably be obliged to face the mystery that the Universe has been created in a remarkably orderly and highly special state after all. One possible approach to this mystery is through the anthropocentric—that if the cosmos were not so regular we could not be here to know about it. A highly turbulent big bang would overproduce entropy and the cosmic background radiation would then be too hot for life to evolve until it had cooled below, say, the boiling point of water. But the temperature of this radiation takes billions of years to diminish appreciably, and if it were substantially hotter than now then the stars would have burnt out before conditions became cool enough for life to form. Without stars such as the Sun there would be no life anyway. This is not, of course, an explanation for the orderly Universe, but it provides an intriguing comment on how lucky we are that the big bang was so well-behaved. □

Red deer or takahe?

from Peter D. Moore

THE conservation of a species normally demands an active management of its habitat in order to increase its carrying capacity for that particular species. Where a species requires a habitat in its early stages of succession, this may mean extensive and frequent modifications of the habitat to prevent its maturation. Generally the amount of management effort declines in situations requiring more advanced successional stages, and for climax ecosystems the major problems are often ones of

prevention of destruction of the habitat, or the control of exotic, invasive predatory or competitor organisms. Experimentation, based upon sound and detailed ecological survey and rational extrapolation, is often necessary before the best available management techniques can be devised and implemented.

Such an experimental approach to conservation is most hazardous where one is dealing with an organism on the brink of extinction. Here one cannot afford to make mistakes. Take, for example, the takahe (*Notornis mantelli*), a flightless gallinule endemic to New Zealand. Subfossil remains of the bird suggest that it once had a fairly extensive range in the south-western tip of the South Island of New Zealand, but the European settlers of the nineteenth century found few specimens and during the first part of this century it was considered extinct. Then, in 1948, it was dramatically rediscovered in the high, alpine and sub-alpine tussock grassland of the Murchison Mountains. It is now known to occur in an area of less than 1,000 km² in these mountains and its population in some parts of this area may still be declining.

Obviously, in such a situation, considerable information is required concerning the environmental requirements of the takahe, before management can be applied. One adverse influence in the area may be the introduced red deer (*Cervus elephas*). Questions such as whether these two species are in competition and whether the deer are modifying the habitat in a manner unfavourable to the takahe, need to be answered.

Mills and Mark (*J. Anim. Ecol.* **46**, 939; 1977) have attempted to provide the information needed to answer these questions. They studied a series of 10 m × 5 m plots in tussock grassland within takahe territory which were situated among the four major dominant tussock-forming plants, *Chionochloa pallens*, *C. flavescentis*, *C. teretifolia* and *C. crassiuscula*. Usage of the plots was assessed by counting the droppings within them, which can be expected to reflect the amount of time spent by takahe in each. Damage to plants (the bird is herbivorous) could also be estimated directly since tussock tillers are pulled off, the base eaten and the remainder discarded in a pile.

This type of study has led Mills and Mark to conclude that the preferred food of the takahe consists of *C. pallens* and *C. flavescentis*, their relative proportions varying seasonally. Plots dominated by these two species were

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also richer in deer faeces, particularly *C. flavescentis*. Chemical analyses of the tillers of four *Chionochloa* species showed that the preferred two species exceeded the others in their concentration of N, P, K, Ca, Mg and soluble sugars (P and K statistically significant). This was not the case for S, Na and starch content. *C. teretifolia* was the species with the lowest overall food value and this species was not recorded as eaten by takahe in the study plots.

From this study it is evident that the two species, *C. pallens* and *C. flavescentis*, are more nutritious and are preferred as food sources by the takahe. The red deer, however, has similar food preferences and this fact, together with the limited abundance of the two plant species (20% and 10% cover respectively) indicates that competition for food may be taking place between the two species. Analyses of individual tussocks which had been selected for grazing suggest that both takahe and red deer selected plants rich in phosphorus for their attention. Overall, the red deer had a more varied diet, including more dicot species, but the coincidence of their preference for phosphorus-rich *C. flavescentis* tussocks confirms an overlap of niche and hence a competitive interaction. Severe competition, however, may be avoided by the fact that deer take the leaf blades whereas takahe take the basal sheaths.

The impact of the competitive interaction is likely to be most severe when some other factor, such as prolonged snow cover in a severe winter, causes harder grazing by the deer and consequent damage to the structure and composition of the alpine grasslands. Under such conditions, of course, the first plants to suffer will be those with the highest phosphorus content, thus depriving the takahe of their nutrient resource.

The New Zealand Forest Service is currently conducting deer population control operations in the area and the work of Mills and Mark suggests that this could have beneficial effects not only for forestry but also for the alpine grasslands and the takahe. For this endangered species, the removal of red deer is likely to do nothing but good.

Order in amorphous polymers

from Paul Calvert

THE problem of whether there is long range order in liquid and glassy polymers has long been troubling polymer science. The 1975 Shrivernham Meeting on Polymer Physics followed closely after a symposium on 'Physical struc-

ture of the amorphous solid state' (*J. macromolec. Sci.* **B12**, 1; 1976) and it seemed that the question was settled; there was no long range order. By the 1977 Shrivenham Meeting the problem had returned, as intractable as ever.

The description of order in liquids and glasses has always been difficult. Bernal's model of dense random packing of liquids was derived by filling a balloon with ball bearings, kicking it round the laboratory, then fixing the structure by pouring in paint so that it could be disassembled and analysed. Regrettably a similar experiment for chain molecules has not been done.

Electron micrographs of thin films of amorphous polymers show 20–50 Å nodules which G. S. Y. Yeh (*J. macromolec. Sci.* **B6**, 451, 465; 1972) claimed to be due to locally ordered regions surrounded by less ordered material. Many microscopists believe these nodules to be artefacts of beam damage, and at the 1975 symposium this straw man was soundly beaten. Small angle X-ray scattering showed unambiguously that there are no significant density fluctuations and small angle neutron scattering demonstrated that the molecules had the same overall radius of gyration as in θ (ideal)-solution suggesting that there could be no ordering. However, semicrystalline polymers have since been found also to have this random coil radius of gyration, so this result must be compatible with order. Yeh has modified his ideas to suggest that the observed blobs are a diffraction effect from regions where the chains are locally parallel. Similar to this is the Pechold meander model (Pechold & Blasenbrey *Koll. Zeit.* **241**, 995; 1970) in which the structure consists of curved, randomly packed bundles of parallel chains. At Shrivenham 1977 it was shown that by folding the bundles correctly a suitable molecular radius of gyration could be produced. There is really no such explicit model of a structure with long range order.

To complement the shortage of models there is also a shortage of methods. Using X-ray diffraction data Warren and Mozzi (*J. appl. Crystallogr.* **2**, 164; 1969) produced a convincing structure for glassy silica based on misalignments of adjacent SiO_4 tetrahedra, but polymers contain more atoms per repeat unit and there is just not enough information in the few broad diffraction peaks that are observed. Wecker, Davidson and Cohen (*J. Materials Sci.* **7**, 1249; 1972) found that the structure in amorphous isotactic polystyrene closely resembled the crystalline state, implying that the chains adopted the same helical structure in each case, and the main change was in the relative arrangement of the helices. Windle and

Lovell (*Polymer* **17**, 488; 1976) have been squeezing more information out of these systems by looking at X-ray diffraction of oriented polymers (this ploy of using fibre patterns is how most crystalline polymer structures have been solved). The spacings of reflections parallel to the chain axes allow the helical conformations to be determined. However, the samples are not really sufficiently oriented, so that it is necessary to sharpen the patterns by analytical methods which might introduce spurious peaks. The results reported at Shrivenham 1977 confirm Wecker's isotactic polystyrene results, propose a predominantly ttgg (t, trans; g, gauche) helix with some tttt for commercial atactic polystyrene which is 70% syndiotactic and suggest a ttg helix for polymethylmethacrylate (Perspex). A rule due to Natta for polymer crystal structures is that intermolecular interactions do not usually distort the chain from the minimum energy conformation of an isolated chain. These results suggest that the predominant helix in amorphous polymers is that found in the crystal structure, so that a similar rule could be proposed that the distribution of conformations in liquid, glass and θ -solvent is the same as that calculated from intramolecular potentials alone. But this still says nothing about how the chains are packed together.

Markova and coworkers claim to have detected regular intermolecular spacings indicative of ordered regions of at least 50 Å by electron diffraction (*J. Polym. Sci. C* **42**, 671; 1973) though

their data analysis has been criticised (Voigt-Martin & Mijchhoff *J. appl. Phys.* **46**, 1165; 1975). At Shrivenham 1977 Longman and Wignall described the level of order in polyethylene as being similar to that in liquid paraffins. Since, however, it is impossible to find two groups in this field who accept each other's data analysis, it is hard to know how firm these conclusions are.

In principle infrared spectroscopy or NMR ought to be able to detect chain conformations associated with kinks in the helix where the chain changes direction, but I am not aware of any such quantitative measurements. Light scattering can detect regions of local orientational order, thus Brillouin scattering and depolarised Rayleigh scattering both support Longman and Wignall's conclusion that polyethylene is no more ordered than paraffin chains of 20–30 atoms (Patterson *et al. Macromolecules* **10**, 667, 737; 1977; *J. macromolec. Sci.* **B12**, 61; 1976.)

If, then, we ask whether chains in the amorphous state adopt the same conformations as in θ -solvent the answer so far seems to be yes. On this basis the length of the straight sections of chain will depend on chain stiffness, in line with the observation that stiff molecules such as polytetrafluoroethylene and poly-4-methyl pentene show particularly sharp X-ray diffraction peaks in the liquid state, implying increased order. We must now ask if it is possible to pack the molecules to the required density without them lying parallel, and so far there is really no answer to this. □

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ILLUSTRATION of penguins (*Eudyptes chrosolopha*) and young on the islands of St Paul and Amsterdam. From an account of an expedition to these islands published in *Archives de Zoologie Expérimentale et Générale* (**6**; 1877).

review article

Handedness of atoms and parity non-conservation

G. Feinberg*

Experiments to test the existence of parity non-conserving interactions in atoms are described. Such experiments have been done in heavy atoms, but their theoretical interpretation is difficult. Prospective experiments in hydrogen and deuterium should clarify the situation, and determine which unified gauge theory can describe weak and electromagnetic interactions.

THE phenomenon of handedness, that is, the existence of physical systems that are not identical to their mirror images, is well known for macroscopic systems, such as the human body. It is also well known that some molecules, such as tartaric acid, occur in two forms with opposite handedness, which can be distinguished from each other by their property of rotating the plane of linearly polarised light in opposite directions¹.

The existence of handed molecules has nothing to do with parity non-conservation, that is, with a failure of the complete molecular energy operator to be invariant under space reflection, although it is conceivable that the prevalence of one type in living things on earth is related to such failure². The two types of molecule are both quasi-stable states, and their existence is the consequence of near degeneracies between opposite parity energy levels of a parity conserving electrostatic potential energy³.

If the atomic potential energy contains some small terms that do not conserve parity, then it is possible that in isolated atoms, where such near degeneracies are not found, there may occur a more profound type of handedness, such that the mirror image of an atomic state is not a stable state at all. This would be analogous to the situation for neutrinos: as far as we know, only left-handed neutrinos occur in nature, and the mirror image right-handed neutrinos do not exist⁴.

If any such handedness occurs in atoms, it must be very small⁵. Furthermore, until about 1968 there was little theoretical reason to expect any parity non-conserving terms in the atomic potential energy. But over the past 10 years, the invention of theories that unify weak and electromagnetic interactions⁶, and the apparent success of such theories in describing neutrino scattering, have provided new stimuli to the search for handedness in atoms.

In these unified theories, the interaction energy between electrons and nuclei originates not only from the exchange of photons, but also from the exchange of massive neutral, spin-1 particles. This new interaction energy, unlike that coming from photon exchange, may not be invariant under space reflection, because the spin-1 Z particles that are exchanged, have as their sources both vector and axial vector currents which behave oppositely under space reflection. The potential coming from Z particle exchange is also very short range because of the high mass of the Z particle, and so involves the small probability of finding an electron at the nucleus of the atom. In terms of the electron wave functions the parity non-conserving interaction may be written as

$$H_{PV} = \frac{G_F}{\sqrt{2}} \sum_i \sum_j \left[(C_{1P} \psi_i \gamma_\mu \gamma_5 \psi_j (\gamma^\mu)_P + C_{1N} \psi_i \gamma_\mu \gamma_5 \psi_j (\gamma^\mu)_N \right. \\ \left. + C_{2P} \psi_i \gamma_\mu \psi_j (\gamma^\mu \gamma^5)_P + C_{2N} \psi_i \gamma_\mu \psi_j (\gamma^\mu \gamma^5)_N \right] \quad (1)$$

In this formula ψ_i are the Dirac wave functions of the electrons in the atom, evaluated at the nucleus, and the sums i, j are carried out over the electrons and the neutrons (N) and protons (P) respectively. The constant G_F is the weak interaction coupling constant ($G_F \simeq 10^{-49}$ erg cm⁻³). C_{1P}, C_{1N}, C_{2P} , and C_{2N} are numbers which vary from one unified gauge theory to another, and are the quantities that theorists would most like to find from a study of atomic handedness. In the standard theory of Weinberg and Salam^{6,7} the values of the C_i are given by

$$\begin{aligned} C_{1P} &= \frac{1}{2}(1-4\sin^2\theta) & C_{2P} &= \frac{1}{2}(1-4\sin^2\theta) \\ C_{1N} &= -\frac{1}{2} & C_{2N} &= -\frac{1}{2}(1-4\sin^2\theta) \end{aligned} \quad (2)$$

The quantity $\sin^2\theta$ must be determined from experiment. The neutrino experiments⁸ are fitted by the standard theory with $\sin^2\theta \simeq 1/4$. In other unified gauge theories, more complex than the original theory, the values of these constants may be different. Indeed, in one class of theories, there may be little or no parity non-conserving interaction between electrons and nucleons. Therefore, the detection and precise measurement of atomic handedness can provide a crucial test of these forms of the gauge theory.

The terms in H_{PV} will have matrix elements between states of opposite parity. The terms involving C_1 are proportional to the number of protons (Z) and neutrons ($A-Z$) in the nucleus, while those involving C_2 are proportional to the spins of the proton and neutron. We shall see that the latter terms are less important in heavy atoms, and in experiments that are insensitive to the nuclear spin alignment. Because each term involves the electron wave functions at the nucleus, the only states for which the matrix elements are not negligibly small are $S_{1/2}$ and $P_{1/2}$ states. In other states, the Dirac wave functions all vanish at the nucleus. Accordingly, H_{PV} will lead to a parity mixing in $S_{1/2}$ and $P_{1/2}$ single electron states. That is, an $S_{1/2}$ state will actually contain a small admixture of various $P_{1/2}$ states and conversely.

The amount of this admixture, which measures the handedness of the atom, will be given, for a specific $S_{1/2}$ state, by

$$\delta(S_{1/2}P) \sim \langle S_{1/2} | H_{PV} | nP_{1/2} \rangle \\ [E_{S_{1/2}} - E_{nP_{1/2}} + (i/2)(\Gamma_{S_{1/2}} - \Gamma_{nP_{1/2}})]^{-1} \quad (3)$$

where E is the energy of the state, and Γ is its decay width.

This mixing can be calculated straightforwardly for a hydrogen or deuterium atom. It is found that in such atoms by far the largest mixing occurs between $S_{1/2}$ and $P_{1/2}$ states of the same principal quantum number, such as $2S_{1/2}$ and $2P_{1/2}$. This is because the energy difference between these states is abnormally small, given by the Lamb shift, which is of order 10^{-6} eV, compared to several eV for the energy difference between states of different principal quantum number. Because of the presence of the nuclear spin dependent terms in H_{PV} , the mixing depends on which of the

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Table 1 Calculations and experimental results on optical rotation in bismuth

Wavelength of transition (Å)	Experiment	Theory, ref. 18	Theory, ref. 21	Theory, ref. 20	Theory, ref. 16
8,757 Å	-0.07 ± 0.32 (ref. 14)	-2.3	-1.2	-2.3	-1.6
6,477 Å	+0.27 ± 0.47 (ref. 15)	-3	-1.6		-1.7

The numbers given are for $10^7 \times \text{Im}M(E1)$. Most of the calculations use the value $\sin^2\theta = .35$. Use of the value $\sin^2\theta = 0.25$ will decrease the theoretical values by a factor of about 0.8.

hyperfine states in hydrogen are being mixed. In the case of zero external magnetic field, the mixing can be written as⁹

$$\delta(2S_{1/2}, 2P_{1/2}) = -i\bar{C}(1.2 \times 10^{-11}) \left(1 + \frac{\Gamma(2S_{1/2}) - \Gamma(2P_{1/2})}{2(E_{2S_{1/2}} - E_{2P_{1/2}})}\right)^{-1} \quad (4)$$

In this equation

$$\bar{C} = C_{1P}Z + C_{1N}(A - Z) - [F(F + 1) - I(I + 1) - \frac{3}{4}] \quad (5)$$

$$(C_{2P}K_P + C_{2N}K_N)$$

where F is the total angular momentum and I is the nuclear angular momentum. K_P and K_N are 2 and 0 in hydrogen, and both 1 in deuterium. The ratio

$$\frac{\Gamma_{2S_{1/2}} - \Gamma_{2P_{1/2}}}{2(E_{2S_{1/2}} - E_{2P_{1/2}})}$$

is approximately 1/20 in hydrogen or deuterium.

It can be seen from equation (4) that if the C_i values are numbers of order 1, as expected in the Weinberg model from equation (2), then the mixing in neutral hydrogen or deuterium is approximately

The small value of δ is a consequence of the very short range of the weak interactions that generate H_{PV} , in comparison with the size of an atom. This range is approximately 10^{-16} cm, and the probability of finding an electron that close to a nucleon in an atom is very small indeed.

This admixture of opposite parity states will induce a small handedness for a hydrogen atom in a $2S_{1/2}$ or $2P_{1/2}$ state. Because of the very small value of δ it seems difficult to measure this handedness in hydrogen atoms without external fields. Two alternative approaches to the measurement of the atomic handedness predicted by the unified weak interaction theories have therefore been proposed.

Heavy atoms

In atoms with large nuclear charge, such as bismuth, with $Z = 83$, the parity admixture δ can become somewhat larger than in hydrogen, for two reasons. One is that the electron wave functions at the nucleus are much greater in magnitude, as even an S-state valence electron is more likely to be found near a heavy nucleus than is an electron in hydrogen. The other reason is that the terms in H_{PV} involving C_{1P} and C_{1N} are proportional to Z and $(A - Z)$, and so are 100 times or so greater than in hydrogen. On the other hand, the nuclear spin dependent terms involving C_{2P} , C_{2N} are not proportional to Z or $(A - Z)$ and so are presumably 100 times smaller in heavy atoms, and negligible as a first approximation. These enhancement effects in heavy atoms are somewhat counteracted by the fact that the energy differences of opposite parity states of valence electrons of heavy atoms are of order 1 eV, rather than the 10^{-6} eV of the $2S$ - $2P$ splitting in hydrogen. Calculations by Bouchiat and Bouchiat¹⁰ indicate a typical value for the mixing coefficient between individual states of opposite parity in a heavy atom such as Bi, of about 10^{-10} , or 10 times greater than the $2S$ - $2P$ mixing in hydrogen.

Two types of experiment have been attempted to detect the parity mixing in heavy atoms. One type, proposed by Bouchiat and Bouchiat¹⁰, is in progress in Paris¹¹ and at Berkeley¹². It

involves comparing the absorption rates of left-circularly polarised and right-circularly polarised photons by the heavy atoms. If the atoms have a slight handedness, these rates will be different. The difference arises because the absorption can, in the presence of handedness, occur both through magnetic dipole ($M1$) and electric dipole ($E1$) transitions, and these two terms interfere to give a difference in the absorption rates. A situation is chosen in which the $M1$ transition would occur even in the absence of parity mixing whereas the $E1$ transition only occurs because of this mixing. In this situation, the absorption rates of the two types of photons are proportional to

$$R_{\pm} \sim (M_{M1} \pm \delta M_{E1})^2 \quad (6)$$

where δ is the mixing, and M_{M1} , M_{E1} are the matrix elements for the $M1$ transition between the unmixed states, and for the $E1$ transition between the admixed state and the unmixed part of the other state. Actually δM_{E1} must be summed over all states that can be admixed into the original states.

The relative difference in the absorption rates is for small δ

$$\frac{\Delta R}{2R} \equiv \frac{R_+ - R_-}{2R_+} \sim 2 \frac{M_{E1}}{M_{M1}} \delta \quad (7)$$

Since M_{E1}/M_{M1} is usually large, this difference is much greater than δ itself, and so is capable of measurement. In the actual experiments in progress, there are additional complications involving static electric fields, which are introduced to help amplify the handedness further, and to distinguish it from background effects.

In the other type of experiments, use is made instead of a relation between the difference of absorption rates for the two circular polarisations of photons, and the angle through which the plane of polarisation of linearly polarised light at resonant frequency is rotated upon passing through a medium consisting of bismuth vapour. This relation is given

$$\Delta\theta \sim \frac{\pi N l (\omega - \omega_0)}{(\omega - \omega_0)^2 + \frac{1}{4}\Gamma^2} |R_+ + R_-| \text{Re}(R_+ - R_-) \quad (8)$$

In this formula, N is the number density of Bi atoms, l the path length through the vapour, ω the light frequency ω_0 the resonant frequency, and Γ the width of the resonance. The path length l is limited by the absorption, so that l cannot be more than a few times the absorption mean free path so that

$$1/l \approx \frac{N |R_+ + R_-|^2 \Gamma 2\pi}{4[(\omega - \omega_0)^2 + \frac{1}{4}\Gamma^2]} \quad (9)$$

From equation (8) and (9) we get

$$\Delta\theta \sim \frac{\omega - \omega_0}{\Gamma/2} \frac{\text{Re}(R_+ - R_-)}{R_+ + R_-} \quad (10)$$

Therefore a measurement of $\Delta\theta$ determines the same quantity as does $\Delta R/R$ in the other type of experiment. It is apparently easier to measure a small rotation angle than to measure a small change in absorption rate, and so the optical rotation experiments have proceeded more rapidly. Two experiments of this type have already been done¹³⁻¹⁵ with results summarised in Table 1.

In order to interpret these experiments, it is necessary to

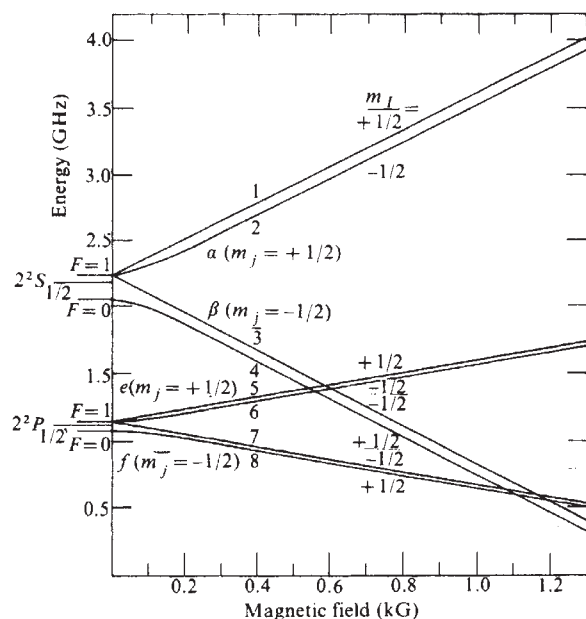


Fig. 1 The Breit-Rabi diagram for hydrogen, with $n = 2$.

calculate R_+ , R_- for the atomic transitions involved. The calculation of M_{M1} , or its direct measurement for the transitions of interest, is not difficult. On the other hand, the calculation of M_{E1} presents substantial problems, even if the values of C_{1P} and C_{1N} are specified. This is because in heavy atoms, no single state dominates the mixing as it does in hydrogen. Instead, a large number of opposite parity states have comparable energy differences from a given state, and are therefore mixed into it by H_{PV} with comparable coefficients. In order to obtain an accurate value for M_{E1} , it is necessary to sum over all such states. We can write

$$M_{E1} = \sum_n \langle i | H_{PV} | \frac{n}{E_i - E_n} \langle n | (E1) | f \rangle + \sum_n \langle i | (E1) | \frac{n}{E_i - E_n} | H_{PV} | f \rangle \quad (11)$$

Here $|i\rangle, |f\rangle$ are the initial and final states, E_i, E_f their energies, $|n\rangle$ are a set of intermediate states with opposite parity to $|i\rangle$, and $(E1)$ is the electric dipole operator

$$(E1) = e \sum_j z_j$$

where the sum is over all electrons in the atom.

Two approaches have been used to calculate this sum. In one, proposed by Bouchiat and Bouchiat¹⁰, and applied to Bismuth by Novikov *et al.*¹⁶, the matrix elements of H_{PV} between certain valence electron states are calculated, and the corresponding matrix elements of $(E1)$ are obtained from experiment. This gives a part of the sum involved in equation (11). Corrections to this coming from other excited states are calculated by various approximations. The results agree fairly well with the other method of calculation, described below (Table 1).

In the second approach, used by Henley and Wilets¹⁷ and by Sandars and collaborators¹⁸, the electrostatic interaction between the electrons is replaced by an average central potential, designed to reproduce the observed energy levels of the states. The difference between this central potential and the true electron-electron interaction is treated as a perturbation. This approximation makes it possible to replace the sum over intermediate states in equation (11) by a sum over single particle excitations. Furthermore, by using a technique invented by Sternheimer¹⁹ and others, it is possible to evaluate the sum directly, in terms of the solution to a differential equation.

$$(H_0 - E)|\psi_1\rangle = (E1)|\psi_0\rangle \quad (12)$$

where H_0 contains the approximate central field, and $|\psi_0\rangle$ is an eigenstate of H_0 with energy E . This is a single particle equation. Once it is solved, $M(E1)$ can be expressed as

$$M(E1) = \langle \psi_1 | H_{PV} | f \rangle + \langle i | H_{PV} | \psi_1 \rangle \quad (13)$$

These expressions now include a sum over all excited single electron states, discrete and continuum. The results of these calculations are presented in Table 1. They can be seen to agree fairly well with each other, and with the results of the other approach.

However, there are various corrections to these results that must be considered, involving the effect of the residual interaction, which is the difference between the true electron-electron interaction and the approximate central potential. The effects of this residual interaction have been considered in two recent papers^{20,21}. Henley *et al.*²⁰ analysed the effect of the residual interaction on configuration mixing of the valence electron states, and found it to be small, of the order of a few per cent in Bi.

The contribution of the residual interaction to excitation of core electrons has been analysed by Loving and Sandars²¹, and found to be quite large, and opposite in sign to the contribution of equation (11). The discovery of this new contribution leaves the prediction of the standard gauge theory for the Bi optical rotation measurements somewhat in doubt. Loving and Sandars believe they have isolated and correctly estimated the main effects of the residual interaction, giving the overall result quoted in Table 1. If they are correct in this belief, there remains a substantial disagreement between the standard model and experiment. Further calculations as well as experiments using other heavy atoms will be needed to clarify this point.

Experiments in hydrogen and deuterium

Partly because of the difficulty of making accurate calculations for heavy atoms, and partly because the heavy atom experiments are relatively insensitive to C_{2P} , C_{2N} , several experimental groups have attempted to detect a handedness of hydrogen and deuterium atoms. In such atoms, the atomic physics is quite simple, and if an effect is detected, it can easily be analysed theoretically.

The expected amount of handedness in these atoms given by equations (4) and (5) is quite small, and it must be enhanced in order to be detected. One way to do this is to use external magnetic fields to decrease the energy difference between $2S_{1/2}$ and $2P_{1/2}$ in certain hyperfine states^{22,23}. There are several values of the external magnetic field in the neighbourhood of 10^3 gauss at which level crossings occur between these states (Fig. 1). At such level crossings, the real part of the energy denominator in equation (3) vanishes, leaving only the imaginary part, which we have seen is only $1/20$ as great. This means that δ will be 20 times greater than at zero external field. Furthermore, the states which cross at a specific value of the external magnetic field are specific superpositions of hyperfine states. Because of this, the largest value of δ at a level crossing will involve definite linear combinations of C_1 and C_2 , and these combinations will vary from crossing to crossing. This allows the important prospect of measuring both C_{1P} and C_{2P} by measurements in hydrogen, and also measuring C_{1N} and C_{2N} by further measurements in deuterium.

An example of what is involved is provided by an experiment now under way at the University of Michigan²⁴. In this experiment a beam of hydrogen atoms in a specific $2S_{1/2}$ hyperfine state is introduced into a cavity in which are present static magnetic and electric fields, as well as a microwave frequency electric field. The static magnetic field is approximately 550 gauss, at which value there is a level crossing between one of the $2S_{1/2}$ hyperfine states and a $2P_{1/2}$ hyperfine state. The microwave electric field is chosen at a frequency to induce transitions between the initial state and the crossed level. The transition could not, however, occur in the absence of parity mixing because it would be $M1$, and not inducible by the microwave electric field.

It does occur in the actual setup by two distinct paths. The external static electric field can mix $2S_{1/2}$ and $2P_{1/2}$, thus allowing an $E1$ transition from $2S_{1/2}$ to $2P_{1/2}$ by the microwave field. This path involves no parity violation. Alternatively, the $2S_{1/2}$ – $2P_{1/2}$ mixing can occur through H_{PV} , followed by the same $E1$ transition. The transition rate involves an interference between these two paths, and takes the form²⁴, for the specific conditions of the Michigan experiment,

$$R = R_0[1 \pm 2.5 \times 10^{-6} C_{2P}]$$

where R_0 is the rate in the absence of H_{PV} . The $\pm$ sign occurs for different relative orientations of the various electric and magnetic fields. Therefore, if C_{2P} is a number of order 1, as suggested by equation (2), the transition rate will vary by about 1 p.p.m. as the fields are reversed. The experimenters believe that they can detect this small variation in a reasonable amount of running time. Similar experiments are being carried out at Yale University and at the University of Washington. In each case it is expected that the experiments can be done in deuterium as well as hydrogen. The Yale experiment will be sensitive to a different combination of C_1 and C_2 . The successful completion of these experiments will then give the values of each of the four constants that determine H_{PV} .

If further calculations and experiments in heavy atoms and in H and D confirm the Bi results, it will be necessary to modify the theory leading to equations (1) and (2). A number of proposals along this line already exist^{25,26}. Some of these new models

predict that C_{1P} , C_{1N} both vanish, but C_{2P} , C_{2N} do not²⁵. These models lead to greatly decreased effects in heavy atoms but results similar to the standard model in H and D. In other models all four of the C_i are very small or zero²⁶, and therefore predict diminished parity violation effects everywhere. The H and D experiments will obviously be very useful in distinguishing between these models. Some results are expected within the next year.

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articles

Silicalite, a new hydrophobic crystalline silica molecular sieve

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A new polymorph of SiO_2 (silicalite, refractive index 1.39, density 1.76 g cm^{-3}) has a novel topologic type of tetrahedral framework. This encloses a three-dimensional system of intersecting channels defined by 10-rings wide enough to adsorb molecules up to 0.6 nm diameter. Silicalite is hydrophobic and organophilic, and selectively adsorbs organic molecules over water.

A MAJOR scientific and technological achievement since 1949 has been the discovery and development of synthetic crystalline, aluminosilicate zeolites as molecular sieve adsorbents and catalysts. We now report the synthesis, crystal structure, and properties of silicalite, a new microporous crystalline silica with remarkable sieve properties. Unlike aluminosilicate zeolites which are hydrophilic, silicalite is hydrophobic and organophilic, and selectively adsorbs organic molecules in the presence of water.

The crystal structure is a new topologic type of tetrahedral framework, which contains a large fraction of five-membered

rings of silicon-oxygen tetrahedra. The framework outlines a three-dimensional system of intersecting channels defined by 10-rings of oxygen ions in all three directions. Organic quaternary ammonium ions which occupy the channels in the precursor obtained by hydrothermal synthesis, are removed by heating to yield silicalite. The resulting void occupies about 33% of the crystal volume, and the three-dimensional channel is wide enough to adsorb molecules up to about 6 Å in diameter. Silicalite can be heated to near 1,300 °C where it degrades to a glass.

Synthesis

The silicalite precursor is crystallised hydrothermally in a closed system containing alkylammonium cations (for example, tetrapropylammonium), hydroxyl ions, and a reactive form of silica at 100–200 °C. The organic-containing precursor crystals have a typical empirical composition $(\text{TPA})_2\text{O} \cdot 48\text{SiO}_2 \cdot \text{H}_2\text{O}$, mean refractive index 1.48, and measured density 1.99 g cm^{-3} . The organic cation is larger than the pore and therefore must be removed by chemical or thermal decomposition (usually calcination in air at 500–600 °C) to yield the microporous silicalite

crystals (mean refractive index 1.39, density 1.76 g cm^{-3}). As the silica framework is electrically neutral, the organic ion is apparently occluded with hydroxyl ions to maintain charge balance, as indicated by infrared spectroscopy. The unit cell composition for the precursor crystals can be expressed as $[4\text{TPAOH} \cdot 96\text{SiO}_2]$. Unlike aluminium-containing zeolites, silicalite has no cation exchange properties.

The crystallisation mechanism of the precursor seems to involve silica clathration of the hydrophobic organic cation analogous to the formation of crystalline water clathrates of alkylammonium salts^{1,2}. Thus the silica tetrahedra assemble into a framework in place of the hydrogen-bonded water 'lattice' of the water clathrate, and surround the hydrophobic organic guest molecules. The alkylammonium ion seems to enhance the solubility of silica in water in a manner reminiscent of the 'structure-breaking' or 'cluster' forming properties of these same ions in aqueous solution³. Indeed, the alkylammonium ion further links the chemistry and structure of water and silica, and seems to translate the structural chemistry of water below room temperature to silica near 200°C .

Crystal structure

Precursor crystals are typically $20 \times 20 \times 70 \mu\text{m}$ elongated along c , and commonly occur as interpenetrant twins on the (110) plane. Crystals calcined to 600°C over 2 d have unit-cell edges a , 20.06; b , 19.80; c , 13.36 Å. The systematic absences ($hk0$, $h = 2n+1$; $0kl$, $k+1 = 2n+1$) indicate space group Pnma or $\text{Pn}2_1a$. Although the symmetry is apparently orthorhombic, weak diffractions may result from either lower symmetry or intergrowth of a second phase. From 8,297 diffraction intensities collected with monochromatised $\text{CuK}\alpha$ radiation out to 2θ 110° with a Picker FACS-I diffractometer, 3,542 unique diffractions were obtained with 764 above background (3σ). The crystal was twinned, and overlap of diffractions was corrected for the 90:10 ratio of the twin volumes.

Combination of direct methods (MULTAN program) and model building yielded the structure in $\text{Pn}2_1a$ (Table 1). The 96 tetrahedra per unit cell form a 4-connected framework with a system of intersecting channels (Fig. 1) composed of near-circular zig-zag channels along a (free cross-section $5.4 \pm 0.2 \text{ Å}$) cross-linked by elliptical, straight channels along b (free cross-section $5.7 \times 5.1 \times 5.2 \text{ Å}$). Both channels are defined by 10-rings. The calculated free cross-section assumes that oxygen ions have a radius of 1.3 Å, and depends slightly on the choice of diametrically-opposing oxygens. The channel system same topology as that reported for a 'shape-selective' has the zeolite⁴, but structural details were not given.

Figure 2A is a photograph down the b axis of a model constructed from tetrahedral stars and plastic linkages. Figure

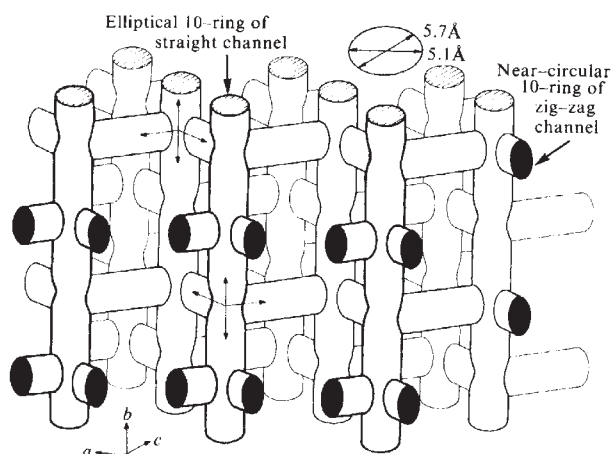
3A is a topological drawing showing how the framework can be constructed from pairs of tetrahedra, 4-rings and corrugated bands of 6-rings all cross-linked by 5-rings and occasional 6-rings. Figure 3B shows the a -axis projection. The corrugated bands of 6-rings are seen end-on, and each 4-ring lies between

Table 1 Atom positions in silicalite

Atom	<i>x</i>	<i>y</i>	<i>z</i>
SI (1)	0.4225 (6)	0.5468 (3)	0.3518 (9)
SI (2)	0.3055 (9)	0.5150 (10)	0.1944 (7)
SI (3)	0.1902 (2)	0.5570 (2)	0.3197 (6)
SI (4)	0.0687 (7)	0.5225 (8)	0.1616 (9)
SI (5)	0.2160 (8)	0.4223 (8)	0.4595 (10)
SI (6)	0.3752 (8)	0.4401 (10)	0.4627 (10)
SI (7)	0.0788 (8)	0.3597 (10)	0.1744 (10)
SI (8)	0.1888 (10)	0.3056 (10)	0.3060 (10)
SI (9)	0.3166 (10)	0.3582 (10)	0.1586 (10)
SI (10)	0.4266 (9)	0.3193 (10)	0.3227 (10)
SI (11)	0.1220 (10)	0.3138 (11)	0.9710 (10)
SI (12)	0.0754 (7)	0.1208 (8)	0.1912 (10)
SI (13)	0.1965 (9)	0.1517 (8)	0.3032 (10)
SI (14)	0.3187 (10)	0.1150 (10)	0.1766 (12)
SI (15)	0.4228 (10)	0.1631 (10)	0.3005 (12)
SI (16)	0.1184 (9)	0.1586 (10)	0.9420 (11)
SI (17)	0.2727 (8)	0.3166 (9)	0.9773 (11)
SI (18)	0.2752 (7)	0.1658 (9)	0.9599 (11)
SI (19)	0.2267 (9)	0.0492 (10)	0.4464 (11)
SI (20)	0.3845 (9)	0.0592 (8)	0.4818 (11)
SI (21)	0.4249 (8)	0.9393 (10)	0.3132 (12)
SI (22)	0.3063 (9)	0.9575 (10)	0.1701 (11)
SI (23)	0.1881 (9)	0.9450 (10)	0.3239 (12)
SI (24)	0.0590 (8)	0.9051 (8)	0.1989 (10)
O (1)	0.3834 (16)	0.3370 (17)	0.2262 (21)
O (2)	0.3730 (17)	0.5271 (18)	0.2454 (22)
O (3)	0.4837 (18)	0.5483 (17)	0.2972 (23)
O (4)	0.2409 (17)	0.5344 (18)	0.2262 (22)
O (5)	0.3047 (18)	0.4414 (19)	0.1849 (23)
O (6)	0.1082 (19)	0.5474 (18)	0.2573 (22)
O (7)	0.1234 (18)	0.3419 (19)	0.2806 (23)
O (8)	0.2527 (17)	0.3213 (18)	0.2209 (24)
O (9)	0.2995 (18)	0.3313 (17)	0.0822 (23)
O (10)	0.0963 (19)	0.3102 (18)	0.0572 (23)
O (11)	0.1306 (17)	0.1508 (19)	0.2555 (24)
O (12)	0.4967 (16)	0.1527 (17)	0.2721 (22)
O (13)	0.3214 (17)	0.0614 (18)	0.1352 (23)
O (14)	0.3687 (17)	0.1690 (18)	0.2261 (24)
O (15)	0.2481 (18)	0.1473 (16)	0.2212 (23)
O (16)	0.3214 (16)	0.1481 (18)	0.0394 (22)
O (17)	0.0836 (16)	0.4454 (19)	0.1702 (24)
O (18)	0.9931 (18)	0.3393 (18)	0.1904 (25)
O (19)	0.4274 (17)	0.2462 (19)	0.3529 (20)
O (20)	0.3866 (16)	0.4978 (18)	0.4116 (25)
O (21)	0.1827 (17)	0.4747 (18)	0.3853 (22)
O (22)	0.4039 (19)	0.3795 (18)	0.4059 (25)
O (23)	0.1907 (18)	0.3629 (18)	0.4021 (24)
O (24)	0.2892 (16)	0.4112 (17)	0.4760 (20)
O (25)	0.1802 (16)	0.4479 (18)	0.5685 (20)
O (26)	0.4021 (17)	0.4694 (16)	0.5966 (21)
O (27)	0.1974 (18)	0.2503 (18)	0.3459 (20)
O (28)	0.2054 (16)	0.3315 (16)	0.9469 (18)
O (29)	0.0696 (15)	0.3590 (16)	0.9334 (20)
O (30)	0.1011 (12)	0.2550 (16)	0.9533 (20)
O (31)	0.2768 (16)	0.2320 (18)	0.9137 (19)
O (32)	0.0818 (17)	0.1548 (17)	0.0798 (19)
O (33)	0.0766 (18)	0.0362 (18)	0.1714 (22)
O (34)	0.2074 (16)	0.1165 (18)	0.4281 (20)
O (35)	0.3970 (16)	0.1289 (16)	0.3892 (21)
O (36)	0.1917 (17)	0.1492 (17)	0.9844 (20)
O (37)	0.0959 (17)	0.1096 (16)	0.8882 (22)
O (38)	0.2978 (18)	0.3715 (17)	0.8828 (23)
O (39)	0.3030 (19)	0.1275 (16)	0.8621 (20)
O (40)	0.1989 (18)	0.0438 (17)	0.5593 (19)
O (41)	0.4144 (18)	0.0564 (17)	0.5818 (22)
O (42)	0.3853 (17)	0.9245 (18)	0.2291 (19)
O (43)	0.2588 (16)	0.9544 (18)	0.2675 (19)
O (44)	0.1171 (16)	0.9156 (17)	0.2414 (19)
O (45)	0.5020 (16)	0.9461 (17)	0.2986 (22)
O (46)	0.3103 (17)	0.0342 (16)	0.4894 (19)
O (47)	0.4198 (16)	0.9896 (17)	0.3833 (22)
O (48)	0.1992 (18)	0.9823 (17)	0.4196 (21)

Values in parentheses following the values for the atom parameters are the error values. The temperature factor was fixed at 1.0 for the silicon atoms and 2.5 for the oxygen atoms.

Fig. 1 Idealised channel system in silicalite. To avoid possible confusion caused by the perspective, the dimensions of the channels along c are shown at upper centre.



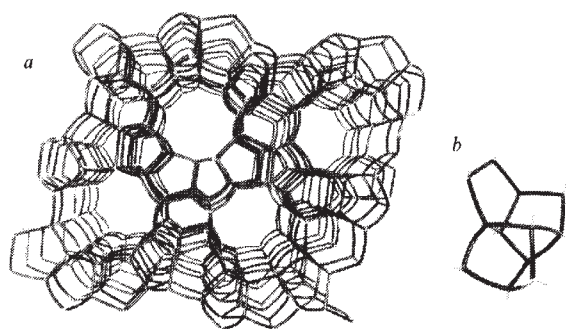
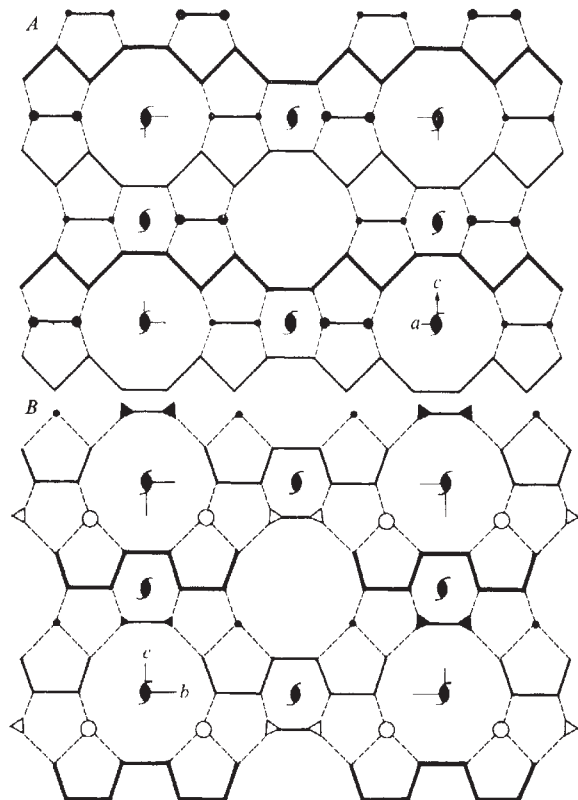


Fig. 2 *A*, Framework viewed down *b*; *B*, Secondary building unit of structure. Twelve tetrahedra linked into five 5-rings and one 6-ring.

two linked pairs. Particularly interesting is the high percentage of 5-rings, a feature shared with the zeolites dachiardite, mordenite, ferrierite and epistilbite. Two other polymorphs of silica, coesite and melanophlogite, also contain 5-rings. The frameworks of the zeolites listed above contain an infinite sheet of linked 6-rings, which might be regarded as a subunit involved in synthesis^{5,6}. Perhaps the corrugated bands of 6-rings may be regarded as a sub-unit in the synthesis of silicalite. Alternatively, the silicalite structure can be assembled from subunits of 12 linked tetrahedra (Fig. 2*B*).

The structural subunits of silicalite can be assembled into other structure types, as will be described elsewhere. These theoretical frameworks give calculated X-ray powder patterns which do not match the observed X-ray powder pattern for

Fig. 3 Topologic drawings of silicalite structure. *A*, *b*-axis projection, *B*, *a*-axis projection. Si atoms lie at the intersections of the lines, and O atoms approximately half-way along the lines. Triple bands of corrugated 6-rings are shown by thick and thin continuous lines. Pairs of tetrahedra and 4-rings occur in projection in (*B*) as dots and circles and as linked open and filled triangles. In (*A*) the pairs of tetrahedra and the 4-rings superimpose as linked dots of two sizes. The corners of the unit cell are shown, together with screw diad axes of symmetry.



silicalite. The present structure gives a calculated pattern which fits well with the observed pattern (Fig. 4). Because of the weakness of intensities from the small crystal used for the structure determination, the *R* factor is rather high at 0.16. The twinning of silicalite around {110} may be due to faulting perpendicular to *b* allowing a pseudo-tetragonal framework that permits *a* and *b* axes to interchange giving the appearance of a (110) twin axis. An 'hourglass' zoning is also seen on the (010) and (100) faces. The calculated density is 1.80 g cm⁻³ in good agreement with the measured 1.76 g cm⁻³.

Adsorption

Silicalite is a molecular sieve adsorbent with an adsorption pore size near 6 Å and a saturation adsorption pore volume of 0.19 cm³ g⁻¹ in agreement with the properties expected from its crystal structure. At ambient temperature, it adsorbs molecules as large as benzene (kinetic diameter 5.85 Å) but rejects molecules larger than 6 Å, such as neopentane (kinetic diameter 6.2 Å). Although the pore-size effect can be used in molecular sieving, its most remarkable adsorption property is surface selectivity. In contrast to the extremely high preference of aluminosilicate zeolite surfaces for water (hydrophilic) and other polar molecules, silicalite has a very low selectivity for the adsorption of water and a very high preference for the adsorption of organic molecules smaller than its limiting pore size. This hydrophobic and organophilic selectivity manifests itself in several ways. Adsorption of organic molecules and permanent gases on silicalite occurs by the volume filling of micropores as in zeolite molecular sieves and other microporous adsorbents. The filling of micropores occurs by physical adsorption at low relative pressures, and is characterised by enhancement of the adsorption energy due to increase of dispersion forces resulting from comparable size of the adsorption volume and the adsorbed molecule. This results in a type I, near rectilinear isotherm as illustrated for the gas phase adsorption of *n*-hexane on silicalite (Fig. 5*b*). Pore filling is essentially complete at a relative pressure of 0.03. In contrast, water does not fill the pores at any relative pressure (Fig. 5*a*). The adsorbed water volume at a relative pressure near one is about 25% of the saturation pore volume for *n*-hexane. In liquid or gaseous mixtures of organic molecules and water, silicalite selectively adsorbs the organic molecule, and thus is capable of removing organic molecules from organic-water streams.

Most solid surfaces are hydrophilic. Previously known hydrophobic surfaces⁷ include graphitised carbon and microporous and macroporous silicas which have been rendered hydrophobic by removal of the hydrophilic surface hydroxyl groups either by thermal dehydration and dehydroxylation or by chemical modification of the surface to replace the hydroxyl

Table 2 Adsorption volumes in silicalite

Adsorbate	Temperature (°C)	Kinetic diameter (Å)	V_p (cm ³ g ⁻¹)	V_t	Molecules adsorbed per unit cell
H ₂ O	RT	2.65	0.047	0.083	15.1
O ₂	-183	3.46	0.185	0.326	38.0
CH ₃ OH	RT	3.8	0.193	0.340	27.6
<i>n</i> -Butane	RT	4.3	0.190	0.334	10.9
<i>n</i> -Hexane	RT	4.3	0.199	0.350	8.8
SF ₆	RT	5.5	0.167*	—	10.9
C ₆ H ₆	RT	5.85	0.134	0.236	8.7
Neo-pentane	RT	6.2	0.029	0.051	1.4

V_p (cm³ g⁻¹) — Total micropore volume in activated silicalite from saturation capacity, calculated using normal liquid densities at the adsorption temperature. Void fraction, $V_t = V_p$ (cm³ g⁻¹) × d_c (g cm⁻³), where d_c is the measured density 1.76 g cm⁻³. All samples activated by calcination in air at 600 °C followed by vacuum activation (10⁻³ torr). Adsorption measurements all by gravimetric McBain-Bakr balance technique. RT, room temperature.

*At 760 torr.

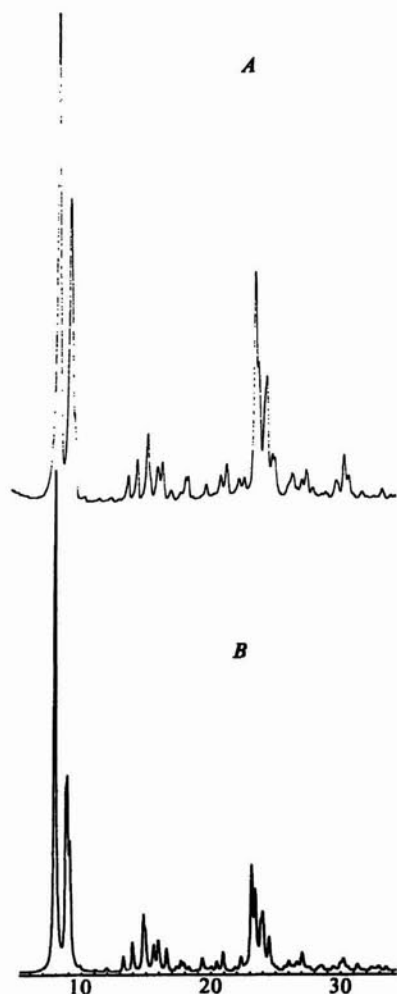


Fig. 4 X-ray powder diffraction pattern of *A*, calcined silicalite material, and *B*, calculated from parameters in Table 1. CuK α radiation with scale in degrees 2 θ .

groups with hydrophobic organic or organosiloxane groups. The water isotherms (Fig. 5A) for Graphon, a dehydrated 'HiSil' silica, and a carbon molecular sieve with a pore size (5.0–5.5 Å) and pore volume (0.20 cm³ g⁻¹) comparable to that of silicalite, suggest that the silicalite surface is similar to or more hydrophobic than the Graphon and dehydrated HiSil surface. The carbon molecular sieve (commercial Pittsburgh activated carbon, Calgon Type MSC-V) is more hydrophilic.

The nature of a hydrophobic adsorbent surface has become understood within the last decade and is reviewed comprehensively in ref. 7. For physical adsorption on an ionic surface, the adsorption interaction energy consists of the sum of dispersion and repulsion energies which originate from non-specific interactions, and electrostatic polarisation, dipole and quadrupole energies which represent specific interactions. For adsorption of water, specific interactions are especially important. In the absence of surface sites which are 'hydrophilic', or of sites for hydrogen bonding, polar or acid-base interactions, the surface becomes nonspecific, homogeneous and hydrophobic. In water, each molecule is hydrogen-bonded to its neighbours (approximately four, as in ice), but in silicalite the narrowness of the channels allows interaction with only about two to three molecules on the average. Because silicalite is electrically neutral, there is no strong interaction with water molecules, and energetically the molecules prefer to remain as a liquid outside the silicalite. What small amount is adsorbed in silicalite is probably associated with the residual hydroxyl groups which persist after thermal removal of the

organic ion in the precursor. The initial isosteric heat of adsorption of water on silicalite is about 6 kcal mol⁻¹, substantially below that of the heat of liquefaction of water (9.7 kcal mol⁻¹), and similar to that reported for Graphon⁷. This requires a high entropy of adsorption, again like Graphon. Low energy and high entropy of adsorption (weak adsorption of highly entropic water) indicate high mobility of the adsorbed water molecule. The strongly hydrophilic nature of aluminosilicate zeolite molecular sieves is due to the presence in the intracrystalline void space of polar groups such as cations and hydroxyl groups, and field gradients generated by the substitution of aluminium for silicon in the tetrahedral framework. Silicalite has no aluminium and no cations in its structure. Chen⁸ has shown a substantial decrease in the amount of water adsorbed on the zeolite mordenite (Zeolon) due to the removal of cations, as well as aluminium from the aluminosilicate framework,

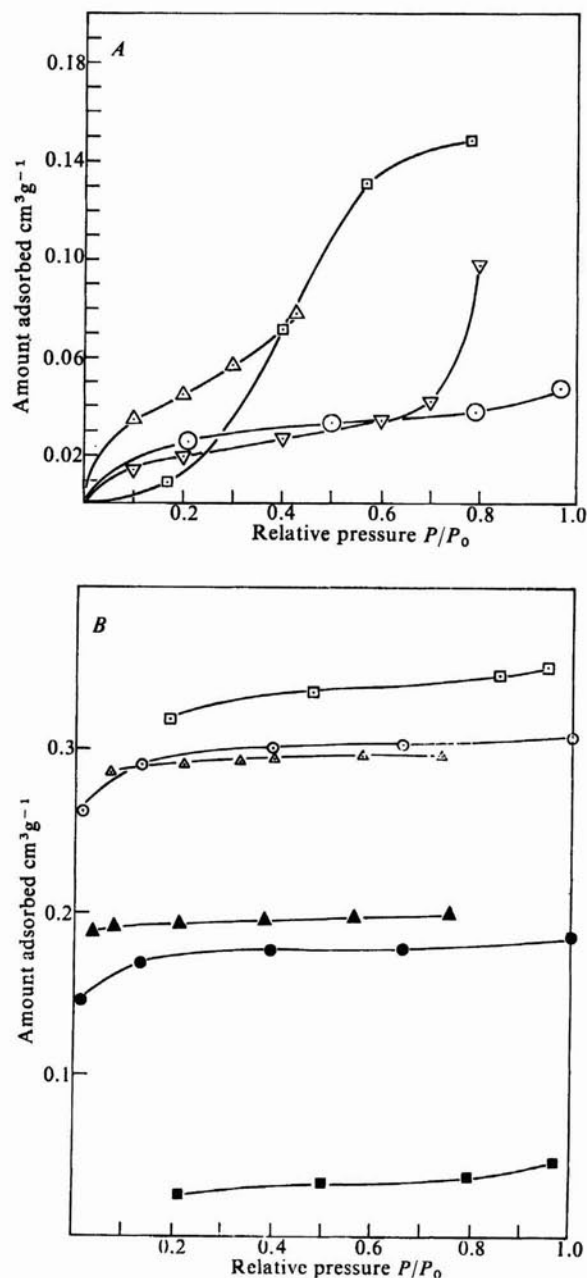


Fig. 5 *A* Water adsorption isotherms on hydrophobic surfaces. □, Carbon molecular sieve; △, Graphon; ▽, Hi Sil; ○, silicalite. *B*, Adsorption isotherms on silicalite and NaX zeolite. O₂ at -183 °C, samples activated at 350 °C, 10⁻³ torr; McBain-Bakr gravimetric measurements. □, H₂O on NaX; ○, O₂ on NaX; △, *n*-hexane on NaX; ▲, *n*-hexane on silicalite; ●, O₂ on silicalite; ■, H₂O on silicalite.

and described the resulting highly siliceous zeolites as 'hydrophobic'.

Adsorption of *n*-hexane in contrast is highly energetic with isosteric heats of adsorption of 16–18 kcal mol⁻¹ over a wide range of relative pressure. This range is substantially above the heat of liquefaction of *n*-hexane (7.8 kcal mol⁻¹), and similar to the isosteric heat of adsorption of *n*-hexane on the molecular sieve zeolite X, again illustrating the high dispersion energy interactions in crystalline molecular sieves where the adsorption cavities and pores are ≤ 10 Å, commensurate with the size of the adsorbed molecule. Consideration of the volume, size and geometry of the void in the silicalite structure (Figs 1–3) and the number and size of *n*-hexane molecules (8.8 molecules per unit cell and 4.5 Å kinetic diameter) shows that the molecules must be highly oriented in nearly linear strings one molecule thick. The fit of *n*-hexane in the channels is near perfect, and *n*-hexane becomes a low entropy highly ordered liquid in the silicalite lattice. Typical adsorption volumes for a variety of molecules on silicalite are given in Table 2.

Stability

Silicalite possesses a remarkable stability for a 33% porous crystal. It is stable in air to over 1,100 °C, and only slowly converts to an amorphous glass at 1,300 °C. It is stable to most mineral acids but reacts with HF similarly to quartz. X-ray emission measurements of the SiK β band show that the mean Si–O bond energy of silicalite exceeds that of quartz by 0.1 eV,

and is essentially the same as for cristobalite. In contrast, the mean Si–O bond energy in aluminosilicate zeolites is substantially less than for quartz⁹.

Applications

Thus silicalite may offer practical applications in the clean-up of water contaminated with organic compounds. Traces of methanol, propanol, butanol, phenol, 1,4-dioxane, pentane and hexane have been removed from water. The selectivity of silicalite is nearest to that of adsorbent carbons, but it has the advantage of much higher stability to regenerative commercial processes involving thermal, acid, and oxidative conditions.

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Radon emanation on San Andreas Fault

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Subsurface radon emanation monitored in shallow dry holes along an active segment of the San Andreas fault in central California shows spatially coherent large temporal variations that seem to be correlated with local seismicity.

RADON is constantly emanated from the Earth into its atmosphere, normally in minute amounts. Radon emanation is known to be anomalously large on active faults and to show temporal variations related to changing atmospheric conditions and possibly nearby seismic activities^{1,2}. To check whether it may show premonitory changes useful for earthquake prediction like those reported for the emanation from deep aquifers^{3,4}, the US Geological Survey began on 7 May 1975 to monitor subsurface radon emanation in 20 shallow dry holes along an active 60-km segment of the San Andreas and Calaveras faults in central California (stations 1–20, Fig. 1). Here I present some initial results of this experiment and discuss possible explanations.

We used a simple track etch method^{5–7} that had been developed for uranium exploration to measure radon emanation: a small piece of plastic film (cellulose nitrate) which is sensitive to α radiation is attached to the inside bottom of a plastic cup (9 cm high, 7 cm aperture). The cup is placed upside down at the bottom of a borehole (10 cm in diameter, 0.7 m deep) to expose the film to the soil gas for 1 week, after which it is replaced by a new cup. The air gap in the cup is sufficient to shield the film from all α particles generated in the soil. As radon and its isotopes are gaseous, however, they may move into the cup to emit α particles close enough to leave tracks in the film. The retrieved film is then chemically etched and the enlarged α -particle tracks in the film within an area of nearly 6 mm² are counted under a microscope. The measured track density is then assumed to be proportional to the average radon

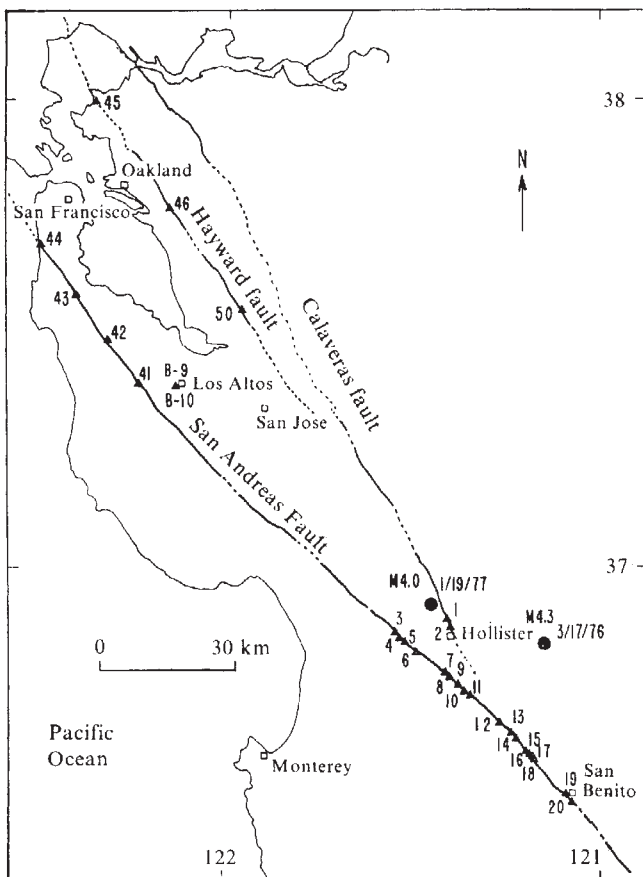


Fig. 1 Location of radon monitoring stations in central California (▲ with station identification numbers) and epicentres of two larger earthquakes (● with magnitudes and dates).

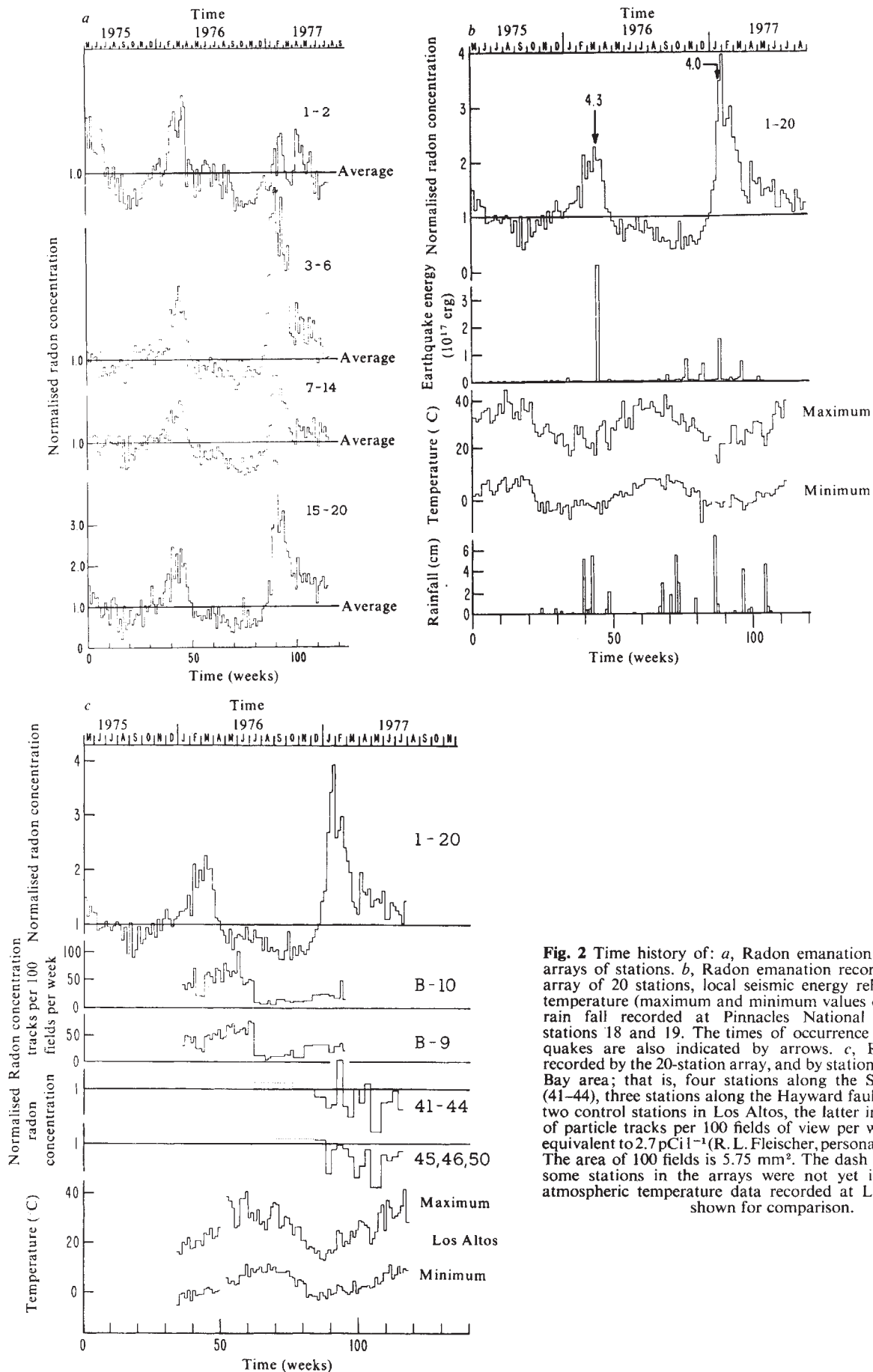


Fig. 2 Time history of: *a*, Radon emanation recorded by four arrays of stations. *b*, Radon emanation recorded by the whole array of 20 stations, local seismic energy release, atmospheric temperature (maximum and minimum values of each week) and rain fall recorded at Pinnacles National Monument near stations 18 and 19. The times of occurrence of the two larger quakes are also indicated by arrows. *c*, Radon emanation recorded by the 20-station array, and by stations in San Francisco Bay area; that is, four stations along the San Andreas fault (41-44), three stations along the Hayward fault (45, 46, 50), and two control stations in Los Altos, the latter in units of number of particle tracks per 100 fields of view per week. Each unit is equivalent to 2.7 pCi l^{-1} (R. L. Fleischer, personal communication). The area of 100 fields is 5.75 mm^2 . The dash lines indicate that some stations in the arrays were not yet in operation. The atmospheric temperature data recorded at Los Altos are also shown for comparison.

concentration of the soil gas in the hole during the period of measurement. In the following, I shall not differentiate between the various contributions to the recorded α radiation by radon and by the isotopes thoron and actinon which have much shorter half lives (55 s and 4 s, respectively) than radon (3.8 d).

The boreholes are supported by plastic pipes 0.8 m long. The upper ends of the pipes are approximately 0.1 m above ground surface and are capped to prevent surface water from flowing into the holes and to reduce possible atmospheric effects. A small amount of moisture was sometimes observed on the retrieved films, especially during the springs for these stations, but it does not seem that the moisture has caused any serious detection problems such as reduced sensitivity, since higher radon emanation was recorded during the springs at these stations. The monitoring sites are located mostly in the fault zones for the purpose of maximising possible tectonic signals, because tectonic strain changes are likely to be amplified in a fault zone due to the mechanical weakness of the zone⁸.

The measured weekly average values of radon concentration of soil gas ranged from 5 to 2,300 pCi l⁻¹. Figure 2a shows radon concentration as a function of time for the 20 stations grouped into four small arrays (stations 1–2 on the Calaveras fault, stations 3–6, 7–14 and 16–20 from north-west to south-east along the San Andreas fault) to improve the signal-to-noise ratio. Data of each array are obtained by first dividing the weekly readings of each station by the average value of the station during the first 40 weeks, and then by summing these 'normalised' values of the same week for all the stations in the array with equal weight.

It is evident from Fig. 2a that the measured radon emanation shows large temporal variations that are spatially coherent over long fault segments (≥ 60 km). The radon variations are not correlated with the relatively small amount of rainfall (Fig. 2b). Also they do not seem to correlate significantly with barometric pressure changes, presumably because of the integrated nature of the measurement, each value representing a weekly average. But because the two prominent peaks in the radon data are separated by nearly a year (46 weeks), one may suspect a seasonal effect, possibly caused by atmospheric heating and cooling of the ground. As shown in Fig. 2b, the correlation between the recorded radon emanation and the atmospheric temperature seems to be exceptionally close. Other observations suggest, however, that this correlation may well be fortuitous. If, for example, the observed temporal radon variations are due mainly to a seasonal effect, one might expect to see fairly similar variations in climatically similar areas, though differences can be caused by different geological conditions at individual sites. Figure 2c shows the radon data recorded by stations that were later installed in the San Francisco Bay area (Fig. 1). These include four stations along a 'locked' segment of the San Andreas fault (41–44), three stations along the Hayward fault (45, 46, 50) and two control stations that are only 3 m apart in Los Altos which is about 8 km off the San Andreas fault. The temperature data recorded at Los Altos are also shown in Fig. 2c for comparison. The radon emanation there apparently varied quite differently, although the temperature variations are similar to those recorded in the 20-station area.

Figure 2b also shows the weekly energy release by earthquakes of magnitude 1.0 and larger within 30 km of the network computed from an energy-magnitude relation given by Richter⁹. The correlation between the radon and the seismicity data seems to be reasonably good. The two largest quakes (magnitude 4.3 and 4.0, respectively, as indicated by arrows in Fig. 2b and solid circles in Fig. 1) occurred near the times when the radon emanation reached its peaks. The emanation began to increase rapidly several weeks before the magnitude 4.3 quake when the smaller quakes did not yet increase in number. This result may indicate that the increase in radon emanation is not due to seismic shaking but may possibly be due to strain build up. In contrast, both radon emanation and seismicity increased more or less concordantly during the second 'anomalous'

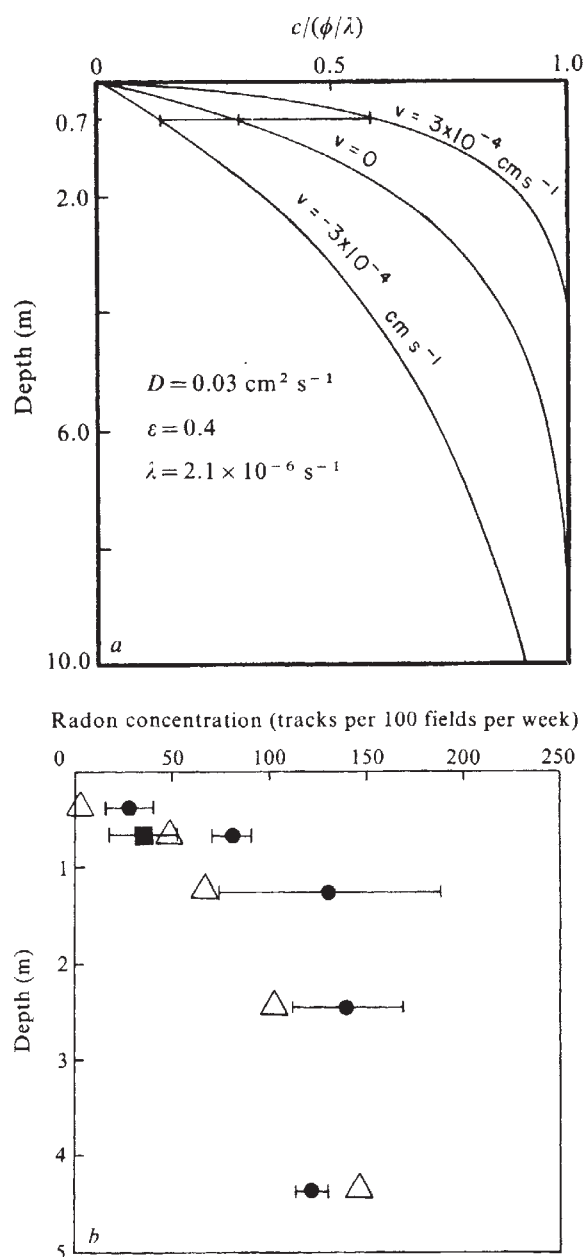


Fig. 3 Radon concentration of subsurface soil gas as a function of depth. *a* for a theoretical model (after Clements¹¹); *b*, observed at site 20. The square indicates a long-term average value at the usual monitoring depth of 0.7 m. ●, Indicate the result for an anomalous period (weeks 96–98) and the triangles the result for a nearly normal period (weeks 99–100). The bars indicate 1σ .

period in which the magnitude 4.0 quake occurred.

From the available data, I suggest that the observed radon variations are more likely to be related to those of seismicity than atmospheric temperature.

If the observed radon variations are indeed mainly earthquake related, what is the responsible mechanism? In view of the fact that radon has a short half life (3.8 d) and moves slowly in the ground¹⁰, it is unlikely that the detected radon comes from earthquake sources several kilometres deep. The anomalously high radon emanation, however, may conceivably be due to an increase in crustal compression that squeezes out the soil gas in the deep reaching fault gouge zone into the atmosphere at an increased rate, as an increased outgassing rate may perturb the vertical subsurface radon concentration profile (radon concentration is known to increase rapidly with depth by about 3 orders of magnitude), such that deeper soil gas containing more radon is brought up to the detection level. (The proposed

mechanism for earthquake related radon emanation variations will be discussed in more detail elsewhere.)

To obtain some quantitative estimate of the effect of a vertical soil gas flow on radon emanation, let us consider the one dimensional model by Clements¹¹. In this model the soil layer is considered to be a homogeneous porous half space (that is the lower boundary is much deeper than 10 m) with uniform radon production. Assume radon migrates vertically by a combination of the following two processes: (1) molecular diffusion governed by Fick's law and (2) gas flow governed by Darcy's law. Having taken into account the equation of continuity for radon and the boundary condition that radon concentration is 0 (negligibly small) at the ground surface, Clements obtained the following steady state result for radon concentration C as a function of depth ($-z$):

$$C = \frac{\phi}{\lambda} \left\{ 1 - \exp \left[\gamma \left(\frac{\epsilon \lambda}{D} \right)^{1/2} z \right] \right\}$$

where

$$\gamma = \frac{v}{2(\epsilon \lambda D)^{1/2}} + \left(\frac{v^2}{4\epsilon \lambda D} + 1 \right)^{1/2},$$

ϕ is radon production rate, λ is its decay constant, ϵ is soil porosity, D is molecular diffusion coefficient of radon in the soil, and v is apparent soil gas flow velocity (volume of flow per unit time per unit geometric area). This result is shown in Fig. 3a for three different flow velocities and some material constants appropriate for typical dry soils. It is evident that a small vertical flow of soil gas (3×10^{-4} cm s⁻¹) can perturb significantly the subsurface radon concentration profile at shallow depths (< 10 m) such that the concentration at the depth of 0.7 m (monitoring depth of this study) is changed by a factor of 2.

To check whether subsurface radon concentration profile varies as predicted, we made radon measurements in boreholes

of several different depths separated by several metres at station 20. Figure 3b, shows the profiles for a 3-week period (week 96–98) during which the radon emanation was still anomalously high (see Fig. 2a), and for a 2-week period (week 99, 100) during which the emanation was nearly back to the average level. It is evident that during the anomalous period radon concentration increased at the shallower depths only, in agreement with the model.

If the observed temporal emanation variations are indeed earthquake related, why are they so large and so spatially coherent for earthquakes that are relatively small? One possibility is that the fault gouge zone, in which most of the radon stations are located, is mechanically much more compliant than the rocks on either side. As a result, strain changes may tend to concentrate in the fault zone over a long segment⁸. Similar spatial coherence is evident in some other fault-zone geophysical data, such as coseismic steps recorded on creepmeters at the times of local earthquakes of comparable magnitudes (> 4.0) (ref. 8) and fault zone deformation episodes indicated by data from alignment arrays¹².

This model may also explain the observed increase in radon emanation resulting from underground nuclear explosions¹³.

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Structural comparison of glycophorins and immunochemical analysis of genetic variants

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Differences in amino acid sequence of erythrocyte membrane glycophorin A are correlated with M or N blood group activity. A second sialoglycoprotein, glycophorin B, has an amino acid sequence identical to that of glycophorin A^N in the first 23 positions and carries N activity only, suggesting that different structural genes code for the glycoproteins carrying these antigens. Certain genetically variant cells lack glycophorin A, as determined by immunochemical methods, and serological MN activity. Other variants lack MN activity, but contain normal amounts of glycophorin A in the membrane.

LANDSTEINER and Levine¹ described a new blood group system in 1927, called MN, in which two alleles *M* and *N* determine the presence of corresponding antigens on the red cells. According to this two-allele theory, three genotypes are possible: *MM*, *MN* and *NN*. In 1947 an additional two-allele antigenic system *Ss* was found²; this is closely linked with *MN* and only rarely have recombination events been observed between *MN* and *Ss*³. The

antigens of the *MN* and *Ss* systems are probably found on different cell surface glycoproteins⁴. There are, however, other data which are inconsistent with this simple genetic interpretation of the serological data. *MM* homozygotes have been shown to express some *N* antigenic activity on the surface of their red cells; this becomes more readily detectable after proteolytic treatment of intact cells⁵. As neuraminidases destroy *MN* activity of human cells, it has been concluded that carbohydrates and sialic acid in particular are essential in the determinants⁶ and that the *MN* alleles code for glycosyltransferases^{7,8}. It has, however, also been shown that various reagents reacting with amino groups destroy *MN* activity and this has led to the idea that protein structures are part of the determinants^{3,4}.

Genetically variant cells have been described serologically, lacking one or the other antigen, and the question arose as to whether absence of the antigen(s) is caused by changes in carbohydrate structure or absence of the glycoproteins carrying the antigenic determinants. We report here studies on the primary structure of glycophorin A isolated from *M* and *N* homozygotes, and the amino terminal segment of glycophorin B. *MN* heterozygotes contain two different glycopeptides in their membrane, which possess different amino acids in positions 1 and 5. The

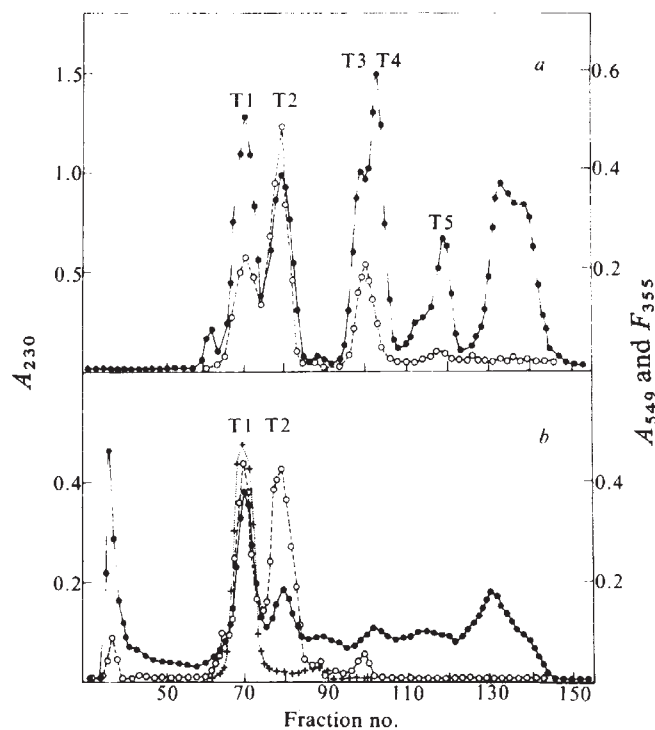


Fig. 1 Separation of tryptic peptides of glycoprotein A (a) and glycoprotein B (b) by gel filtration on Sephadex G150 superfine. 300 mg of glycoprotein A or glycoprotein B (fraction B in Fig. 1, ref. 21) isolated from pooled blood was dissolved in 30 ml of 50 mM Tris-HCl, pH 8.5 and was digested for 24 h with trypsin (TPCKT, Worthington) at an enzyme:substrate ratio of 1:30. After digestion, 3 mg of tosyl-L-Lys-CH₂Cl was added and the pH was adjusted to 4.5 with 1N HCl. The precipitate which formed during acidification was removed by centrifugation and the supernate, containing the soluble peptides, was freeze-dried and after resolubilisation was applied to a column of Sephadex G150 superfine (Pharmacia, Sweden), equilibrated in 0.1 M ammonium acetate at pH 6.8. The column dimensions were 2.4 cm $\times$ 150 cm, flow rate 10 ml h⁻¹, fraction volume approximately 5 ml. The column effluents were monitored for protein by measuring the absorbance at 230 nm (●), for sialic acid content by the procedure of Warren¹⁴ at 549 nm (○) and for tryptophan by measuring fluorescence emission at 355 nm (excitation 295 nm), (+).

glycopeptide isolated from glycoprotein B shares the amino acid sequence of glycoprotein A^N regardless of the MN type of the cell of origin. We further confirm previous suggestions that certain genetic variants at the MN locus entirely lack glycoprotein A⁹⁻¹¹, but genetically determined loss of MN activity is not necessarily correlated with the absence of glycoprotein A.

Glycoprotein A^M and glycoprotein A^N have different amino terminal sequences

In previous studies on the primary structure of glycoprotein A, two positions in the sequence consistently gave two amino acids: leucine and serine as the N-terminus and glycine and glutamic acid in position 5. In view of recent controversial findings⁴¹ and suggestions that glycoprotein A from M or N homozygous individuals contains predominantly one N-terminal residue, namely serine or leucine^{18,19}, we have reinvestigated the N-terminal structure of glycoprotein A isolated from two to five individuals, each with genotypes MM, MN, or NN.

Glycoprotein A was isolated by the LIS-phenol method²⁰ and gel filtration in detergent²¹. After tryptic digestion, the insoluble peptide portion was removed^{13,14} and the soluble peptides were separated by gel filtration (Fig. 1a)¹⁴. As described previously, two glycopeptides (T1 and T2) derived from the N-terminal end of the polypeptide chain, were found^{12,14,15}. Peptide T2 is obtained by cleavage at a lysyl and/or arginyl bond of part of the glycoprotein A molecules within the T1 sequence, which is insensitive to trypsin in T1. T2 is thus shortened at the C-terminal end by nine amino acid residues. T1 and T2 were re-

chromatographed by ion-exchange chromatography¹⁴. The yield of combined peptides T1 and T2 for each preparation was approximately 300 nmoles per unit of blood. The amino acid and carbohydrate composition of some of the peptides are given in Table 1. According to these data, T1 (not shown) and T2 from N homozygotes lack glycine, but contain one additional glutamic acid and leucine residue. The same peptides isolated from glycoprotein A from MN heterozygotes seem to be a mixture of both forms and the amino acid composition is comparable to that found in earlier studies^{12,14}. The carbohydrate composition of T1 (not shown), or T2 is rather similar for the three different preparations (Table 1).

The partial amino acid sequence was determined for the first 7 amino acid residues of T2 by simultaneously using the direct Edman degradation and dansyl-Edman procedure^{12,15}. The amino terminal sequence of glycoprotein A^M is Ser-Ser-Thr-Thr-Gly- as compared to glycoprotein A^N which has the sequence Leu-Ser-Thr-Thr-Glu-. The presence of both serine and leucine in position 1 and glycine and glutamic acid in position 5 for T2 isolated from glycoprotein A^{MN} indicates that this preparation is a mixture of the two types.

PTH-derivatives were not found in any preparation in steps 2, 3 and 4, and as described earlier, the finding of dansylated serine and threonine derivatives in these positions indicates that these residues are glycosylated^{12,15}. Figure 2 also contains the rest of the amino acid sequence of T2, which should be identical for all three preparations, since the amino acid composition does not indicate further heterogeneity and none was observed in this region of the sequence in our earlier studies^{12,15}. Quantitation of PTH-derivatives by gas chromatography was possible only for the non-glycosylated amino acids and we found for A^M-T2 90 nmol of serine in position 1, 13 nmol of glycine in position 5 and 64 nmol of valine in position 6. The uncorrected values of the amino acids found in these positions for A^N-T2 are 75, 39, and 23. No other amino acids were found, indicating that the isolated peptides are homogeneous with respect to the protein structure.

Table 1 Amino acid and carbohydrate analysis of N-terminal peptides of glycoprotein A and glycoprotein B*

	A ^M T2	A ^N -T1	B ^{MN} -T2
Asp	2 (2)	2 (2)	1.3(1)
Thr†	7 (7)	7.1(7)	7.2(7)
Ser	8.7(9)	7.9(8)	8.3(8)
Glu	1.2(1)	2.3(2)	4 (4)
Pro	—‡	—	0.2(-)
Gly	1 (1)	—	2.2(2)
Ala	1.1(1)	1.1(1)	1.2(1)
Cys	—(-)	—	—(-)
Val	2 (2)	2.1(2)	2.7(3)
Met§	0.4(1)	0.4(1)	0.6(1)
Ile	0.9(1)	1 (1)	0.9(1)
Leu	—(-)	1.1(1)	2 (2)
Tyr	0.7(1)	0.8(1)	0.6(1)
Phe	—(-)	—(-)	—(-)
His	2 (2)	2 (2)	1.7(2)
Lys	2 (2)	1.9(2)	1.2(1)
Arg	0.5(-/1)	0.6(-/1)	0.7(1)
Total	30	30	35
Carbohydrate composition			
Fuc	0.6	0.2	—
Man	2.8	2.6	—
Gal	11.9	10.5	10.7
GalNac	8.3	7.6	13.8
GlcNac	5.4	5.1	—
Nana	9.8	10	15.8

*Amino acid analysis was done on a Durrum D500 analyser, using norleucine as an internal standard. The values are given in mol per mol peptide with expected values in brackets.

†Values for Thr and Ser are corrected for losses during hydrolysis by extrapolation to zero time after 24, 36, and 48 h hydrolysis.

‡Dash denotes 0.1 or less.

§Uncorrected values for methionine.

||Mol per mol peptide. Carbohydrates were determined as trimethylsilyl derivatives of methyl glycosides by gas-liquid chromatography, on a Hewlett-Packard 5110 B gas chromatograph, using inositol as an internal standard¹².

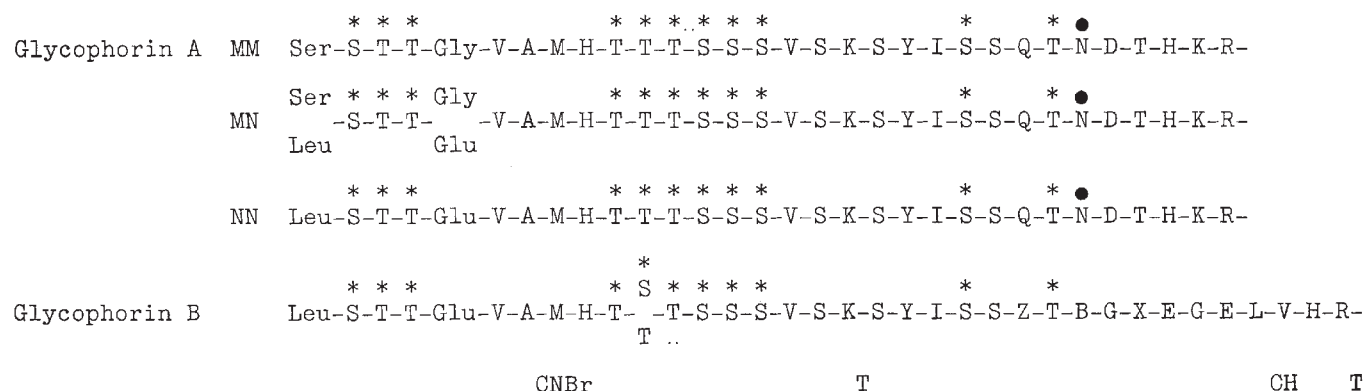


Fig. 2 Amino acid sequence and sites of glycosylation (*●) of the N-terminal region of glycophorin A and glycophorin B. ↑ cleavage sites: cyanogen bromide (CN Br), trypsin (T) or chymotrypsin (CH). - - - residues identified after dansyl-Edman degradation by thin layer chromatography. → residues identified as PTH-derivatives by gas-liquid chromatography. Except from positions 1 and 5, the one later code for amino acids is used⁴³.

N-terminal sequences of glycophorin A^N and glycophorin B are identical

Glycophorin B is not yet as well defined as glycophorin A. This sialoglycoprotein can be distinguished by its different electrophoretic mobility on SDS-polyacrylamide gels^{23,24}, amino acid composition²¹ and antigenic properties (refs 16, 17, and unpublished observations) and can be separated from glycophorin A by gel filtration in detergents²¹ or elution from SDS-polyacrylamide gels¹⁶. In certain genetically variant cells which lack both serological Ss activity and the trace N activity normally associated with MM homozygotes^{5,16}, periodic acid Schiff (PAS) staining bands in the glycophorin B positions are absent²⁹.

It has not yet been possible to isolate electrophoretically pure glycophorin B in preparative amounts. We decided therefore to use fractions B and C (Fig. 1 in ref. 21) obtained by gel filtration of the entire sialoglycoprotein fraction, as starting material for the isolation of glycopeptides. These fractions are highly enriched for glycophorin B, but contain variable amounts of a third and possibly other minor sialoglycoproteins, as suggested previously (refs 22–24, and unpublished observations). Figure 1b shows the chromatogram of the soluble peptides of a tryptic digest of fraction B. The two glycopeptides which elute in this system at positions identical to T1 and T2 of glycophorin A, were further purified by ion-exchange chromatography on DEAE-cellulose in 50 mM sodium formate, pH 6.1, and were eluted with a linear salt gradient (0–0.3 M NaCl). Under these conditions, peptides T1 (or T2) from glycophorin A are separated and thus contamination of the glycophorin B preparation with glycophorin A can easily be monitored.

Peptide B-T2 was also isolated as the major peptide component from a tryptic digest of fraction C (see Fig. 1 in ref. 21), which contains almost pure glycophorin B (data not shown). The amino acid and carbohydrate composition of glycopeptide B-T2 is given in Table 1, which shows that B-T2 is closely related to A-T2 in amino acid composition, but lacks mannose and N-acetyl glucosamine entirely. In contrast, B-T1 contains tryptophan, which is not found in glycophorin A, has an amino acid composition quite different from that of A-T1, but contains mannose and N-acetyl glucosamine (not shown). Further work will be required to establish whether it is derived from glycophorin B or yet another sialoglycoprotein.

We have determined the amino acid sequence of B-T2 by the methods described above. In order to accomplish this, B-T2 was desialated with neuraminidase and was cleaved with cyanogen bromide, trypsin and chymotrypsin^{12,14}, and the peptides indicated by arrows in Fig. 2 were obtained. A more detailed description of these data will be published elsewhere. The secondary peptides as well as B-T2 (steps 1–16) were subjected to manual Edman degradation and we found an amino acid sequence which was identical in at least the first 23–25 amino terminal residues to

glycophorin A^N, despite the fact that this peptide was obtained from glycophorin B isolated from pooled blood of many individuals. Also, the threonine and serine residues glycosylated in glycophorin A were found to be glycosylated in exactly the same positions within the first 25 amino acid residues. B-T2 contains one asparagine or aspartic acid. The lack of glycosylation at this position could be explained either by finding only aspartic acid in this position or by the drastic change in amino acid sequence C-terminal of residue 26, which is entirely different from the corresponding region of glycophorin A. N-glycosylation as far as known invariably occurs at asparaginyl residues in the sequence Asn-X-Thr (or Ser) (ref. 25). On occasion we found serine in addition to threonine in position 12. Since the residue in this position is glycosylated, only dansylation detects an amino acid. The lack of quantitation does not allow us to decide whether serine is the only amino acid residue at this position.

Serological MN activity correlated with amino terminal structure of glycophorins

As indicated earlier, many laboratories have reported the presence of N-activity on MM homozygous cells^{5,7,10}, and this finding is one of the crucial elements for a hypothesis that the N-blood group substance represents the precursor for M⁷. We therefore studied the blood group activities which are associated with these proteins, isolated from individuals which are classified according to the two allele model into MM, MN and NN. First attempts focused on the activities of glycophorin A and glycophorin B preparations isolated as described above. A^N and B^N did not inhibit the agglutination of MM erythrocytes, but B^M inhibited the agglutination of NN erythrocytes, suggesting that MM homozygotes contain N activity in one of the minor sialoglycoproteins as previously suggested⁴. However, the difference between the activity of B^M in inhibiting MM- and NN-agglutination was only 3–4-fold, suggesting that B^M is contaminated with A^M, for which our chemical studies provided sufficient support.

In view of the difficulties in isolating pure glycophorin B, we analysed the serological activities of the tryptic glycopeptides which can be purified and characterised more readily. In Table 2 the data obtained by a semiquantitative haemagglutination-inhibition assay are summarised and the results can be stated as follows: the total glycophorin fraction, glycophorin A and the N-terminal peptides isolated from glycophorin A inhibit the homologous agglutinating system, but not the heterologous system; that is, peptides isolated from M homozygotes inhibit only the agglutination of MM cells, but not of NN cells. Peptides prepared from glycophorin A^{MN} are equally active in both systems; only the amino terminal glycopeptides are active, but not the only other glycopeptide T3A derived from glycophorin A, which is also located at the exterior of the cell^{12,14}; peptides T1 and T2 are equally active, but are 2–4-fold less active than glycophorin A,

Table 2 Serological activity of sialoglycopeptides isolated from glycophorin A and glycophorin B in individuals homozygous or heterozygous at the *MN* locus

Inhibitors	Haemagglutinin-inhibition of inhibitors*	
	MM cells anti- <i>M</i> serum†	NN cells anti- <i>N</i> serum†
MM Glycoprotein fraction‡§	1.5	> 36
A ^M §	2.1	> 25
A ^M -T1	9.4	> 226
A ^M -T2	10.8	520
A ^M -T3A¶	> 2,250	> 2,250
A ^{MN} -T1	n.t.	n.t.
A ^{MN} -T2	20.6	13.7
NN Glycoprotein fraction§	> 40	1.2
A ^N §	> 30	3.7
A ^N -T1	> 90	7.5
A ^N -T2	> 160	6.7
B ^M	6.2	6.2
B ^{MN} -T1¶	> 384	> 384
B ^{MN} -T2¶	> 440	6.9

Sialoglycoproteins and glycopeptides were assayed by a semi-quantitative haemagglutination-inhibition assay. One drop each of serum appropriately diluted (four agglutinating units) was incubated with one drop of a serial dilution of inhibitor for 3 min, before one drop of a 5% erythrocyte suspension in phosphate buffered saline was added. The plates were shaken at room temperature and were scored after 10 min. The protein concentration of the inhibitors was determined by amino acid analysis using norleucine as internal standard.

*Concentration of inhibitors in nmol ml⁻¹ completely inhibiting four agglutinating units. n.t. not tested.

†Sera obtained from Ortho, Lot No. M 127, M 173, and N 157.

‡MM, MN, and NN refer to the serological blood type of the red cell membranes, which were used to isolate the total sialoglycoprotein fraction, glycophorin A (A^M or A^N) or glycophorin B (B) by methods described elsewhere^{20,21}. Tryptic sialoglycopeptides T1, T2, T3A were prepared from glycophorin A or glycophorin B (A-T1 etc., B-T2 etc.)

§Single donors.

||Pooled blood from two donors.

¶Pooled blood from about 25 donors.

which is about 1.5- to 3-fold less active than the unseparated mixture of sialoglycoproteins; only the amino terminal peptide B-T2 of glycophorin B shows activity and this activity is restricted to *N*. The starting material for the isolation of peptide B-T2, glycophorin B, has activity for *M* and *N*, consistent with the phenotype of the original cells from which it has been isolated and assuming that glycophorin A is present in this fraction as a contaminant.

Genetic variants which lack glycophorin A

In Table 3 sialoglycoprotein profiles of membranes from some variant cells are calculated from SDS-polyacrylamide gels²⁶ in comparison with normal cells. The cells we have used are either homozygous or heterozygous at the major *MN*-locus and according to serological data express either no *MN* activity or only half the amount³. On the basis of this analysis, several cell types can be distinguished: heterozygous *M^kN* cells which serologically express only *N*, but no *M* activity, are indistinguishable from normal cells; heterozygous *M^kM* cells³, which serologically express only half the activity of *MN* and *Ss* antigens, have only 50% PAS-stainable protein as compared to normal cells, affecting both glycophorin A and B; cells from two individuals designated *En(a-)* G. W. (Finland) and *En(a-)* E. H. (England) are indistinguishable from each other and show a reduction of the total stainable glycoprotein of about 80%, mostly accounted for by loss of glycophorin A stainability; cells with *Tn* phenotype^{3,28,42} again show about 80% reduction of stainable glycoproteins, which affects both glycophorin A and B. Although these results are in general agreement with serological results (with the exception of *En(a-)* E. H. (refs 30, 40)) and with the finding of others, several problems inherent in this technique should be pointed out.

Tanner and Anstee⁹ first suggested that the major sialoglycoprotein is absent in *En(a-)* cells. Dahr and colleagues in a series of papers concluded from their analysis of gel electrophoretic data (similar to ours) in conjunction with serological results that the serological abnormalities observed in these and other variant cells are correlated with the partial or total absence of one or more of the cell membrane sialoglycoproteins^{10,29,30}. Gahmberg *et al.*¹¹, in a more careful analysis of cells with *En(a-)* phenotype, used various radioactive probes to detect cell surface alterations and arrived at similar conclusions. These data, however, left some doubt, since absence of sialic acid and/or galactose from the glycoproteins would result in loss of just the structures which are required for detection by the techniques used. The attempt to label radioactively the protein backbone of glycophorin A and B by iodination gave equally ambiguous results, since glycophorin B could not be labelled in *En(a-)* cells despite the fact that this protein is labelled in normal cells and was found to be present in these cells by other techniques¹¹. In addition, the interpretation of data obtained by SDS-polyacrylamide gel electrophoresis is complicated by the lack of incomplete separation of glycophorin A and B from each other and other undefined glycoproteins^{21-24,26}, and is not entirely resolved by the use of other electrophoresis systems^{23,27}. Also these proteins tend to aggregate and to migrate electrophoretically at several positions, making quantitation difficult.

In order to overcome these problems we have developed a simple radioimmunoassay procedure which uses rabbit antibodies specific for a C-terminal determinant of glycophorin A. Since these antibodies react with the protein portion of glycophorin A exclusively, post-translational modification of the proteins on the N-terminal segment (which apparently is required for the expression of *MN* activity) or absence of glycosylation should not affect the assay. By using this assay the amount of glycophorin A for normal, unmodified human erythrocyte membranes was determined as 1.6% of the total membrane protein. Removal of sialic acid does not affect the results. Since the reaction of antibodies with glycophorin A can be specifically inhibited by small C-terminal peptides derived from glycophorin A (ref. 17 and unpublished results) and as membranes prepared from erythrocytes of other species, like guinea pig, sheep, or rat do not inhibit binding, no other membrane proteins seem to react or cross-react. Only chimpanzee cells were found to be reactive and contain about the same amount of glycophorin A or an analogue (unpublished) in accordance with the expression of *MN* activity in these cells³¹. When the variant cells listed in Table 3 were analysed in addition to membranes from one individual of the Finnish family heterozygous for *En(a-)*, the results shown in Fig. 3 were obtained. Three groups of cells can be distinguished: cells with no detectable glycophorin A, homozygous *En(a-)* G. W. or E. H.;

Table 3 Polyacrylamide gel electrophoresis and periodic acid-Schiff (PAS) staining of red cell membrane sialoglycoproteins* in genetic variants

Source of membranes	% of control membranes		
	Glycophorin A (PAS 1 & PAS 2)	Glycophorin B (PAS 3)	Total (PAS 1, 2, 3)
<i>M^kN</i>	98.4	101.1	97.2
<i>M^kM</i> (H.S.)	58.8	49.1	57.8
<i>M^kM</i> (F.Q.)	58.2	43.5	56.6
<i>En(a-)</i> (G.W.)	10.9	73	18.6
<i>En(a-)</i> (E.H.)	14.1	76.6	21.2
<i>Tn</i>	19.4	21	19.6

*Membranes were prepared by standard procedures³⁶ and dissolved immediately in buffer containing 5% sodium dodecyl sulphate. After incubation for 30 min at 37°C the samples were applied to 5.6% polyacrylamide gels²⁶ and run until the tracking dye reached 8 cm. The gels were fixed, stained with PAS reagent, and scanned at 560 nm in 0.1% Na₂S₂O₅ in 0.01 N HCl. The peak areas were calculated by triangulation and normalised to equal protein loads. The data from three determinations obtained for variant cells are given in per cent as compared to normal cells for glycophorin A (PAS 1 and 2)²¹, glycophorin B (PAS 3)²¹, and total PAS-stainable protein recovered. For explanation of the variant cells see text.

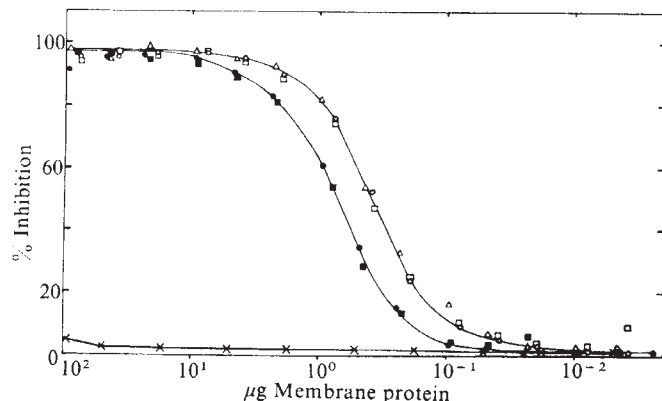


Fig. 3 Analysis of variants at the *MN* locus by quantitative radioimmunoassay-inhibition. Erythrocyte membranes from normal or variant cells (freshly prepared or kept frozen at -70°C) were dissolved in phosphate buffered saline, containing 0.02% NaN_3 and 0.15% Ammonyx-LO (lauryl dimethyl ammonium oxide) and were used in a radioimmuno-inhibition assay as described previously¹⁷. The protein concentration of the inhibitors was determined by amino acid analysis using norleucine as an internal standard or by the Lowry procedure with bovine serum albumin as the standard. Erythrocyte membranes from individuals with genotype M^sN ($\circ$), M^sM (H.S. $\bullet$, F.Q. $\blacksquare$), $En(a-)$ homozygous (G.S. or E.H. $\times$) and two control individuals ($\square$, $\triangle$). *Tn* cells were indistinguishable from normal controls and $En(a-)$ heterozygous cells were identical to M^sM cells in this assay.

cells which contain 50% glyophorin A in comparison to normal, M^s heterozygotes or $En(a-)$ heterozygotes (not shown); and cells which are indistinguishable from normal controls, M^s heterozygotes or *Tn* cells (not shown).

Conclusions and perspectives

The *MNSs* locus determines two proteins, glyophorin A and glyophorin B, which are structurally related. The two alleles *M* and *N* determine a difference in amino acid sequence of glyophorin A, but this difference is restricted to two positions only. Serine and glycine are found in positions 1 and 5 of the amino acid sequence in glyophorin A^M and leucine and glutamic acid replace these amino acids in the corresponding positions in glyophorin A^N. This finding explains the heterogeneity previously observed in these two positions of the amino acid sequence of glyophorin A^{12,14,15}. Glyophorin B probably carries the antigenic determinant(s) which distinguish two allelic forms *S* and *s*, and was found to be identical in sequence with A^N in at least the first 23 residues regardless of the *MNSs* type of the cell membranes, from which the protein has been isolated. As the amino terminal peptide from glyophorin B reacts equally well with antibodies to glyophorin A^N, we conclude that the serological distinction between A^M and A^N resides within the amino terminal region of these proteins. This observation is consistent with the finding that red blood cells from *MM* homozygotes react with anti-*N* sera, particularly when treated with proteases, or are capable of removing antibodies from such sera in absorption experiments. The sialoglycoproteins determined by the *MNSs* locus are thus closely related, but not in the sense postulated by Springer^{7,32}, who proposed that the immunodeterminant structure of *M* and *N* is an oligosaccharide. The two structures were considered to be related since *N* was further postulated to be the biosynthetic precursor for *M*, which is generated by the addition of one sialic acid residue. According to this idea, *M* and *N* are genes which are responsible for post-translational modifications, but not structural genes as shown here. Our finding also does not favour the view that electrostatic interactions between ϵ -amino acid groups of lysine and carboxyl groups of sialic acid are important for a certain steric arrangement of the *M* and *N* specific structures³³. These ideas were based on the effects of modification of the protein by various compounds³⁴ and the changes of activity by removal of sialic acid or by amidation of the carboxyl group of sialic acid residues³⁵. The only two lysyl residues in the amino terminal glycosylated region are found in positions 18 and 30, and

glycosylation in the vicinity seems to interfere with proteolytic degradation by trypsin and chymotrypsin, probably due to steric hindrance or to certain conformational arrangements of the polypeptide backbone, which make these residues inaccessible. The lysyl residues, however, are separated by three and four amino acids, respectively, from oligosaccharide structures. Interaction between different subunits of glyophorin³⁶ could bring such residues in close proximity, but this does not seem to be likely, because small glycopeptides are only slightly less active than the intact protein in its isolated form. Alternative possibilities to explain the above findings are: a free amino group of the N-terminal serine or leucine may be important, as *M* or *N* activity can be restored after removal of the modifying groups³⁴; clustered oligosaccharides and sialic acids may impose certain restrictions to free mobility of the underlying polypeptide backbone. Removal of sialic acid could cause a change in the conformation of the protein, which is accompanied by loss of antigenic activity. It has been suggested that carbohydrates may influence or support certain protein conformations³⁷. More direct studies will be required to solve this question.

Serological studies in humans reveal abnormalities of expression of the *MNSs* antigens in certain rare instances³. Such abnormalities are correlated sometimes but not always with a considerable decrease in the amount of sialic acid associated with the plasma membrane. These observations are reminiscent of studies in mice in which strain differences in electrophoretic mobility and agglutinability are most easily explained by assuming one locus with only two alleles, *Eam^h* and *Eamⁱ* (ref. 38). The overall agreement between the distribution of the phenotypes in different crosses and the expectations from a simple diallelic system were sufficient to assume that one gene is of either unique or overriding importance in determining the surface charge of mouse erythrocytes. It was postulated that one gene either regulates the expression of a sialoglycoprotein directly or that it controls the quantity of a sialyl transferase.

In this study we have shown unambiguously that glyophorin A either can be totally absent from human erythrocytes or that the protein is altered so extensively that it can no longer be recognised as such; a total absence of glyophorin B is also possible²⁹, but no homozygous individual has been found (yet), who lacks both proteins. Loss of *MN* activity, however, is not necessarily associated with the absence of glyophorin A, as the two examples *Tn* and *M^s* show. For *Tn* cells a block in transfer of galactose is postulated^{28,42}. In *M^s* cells, as well as possibly in other variant forms³, the alteration seems to be much more subtle.

The absence of glyophorin A or B is not apparently associated with disease and to our knowledge no functional or other abnormalities have been described involving cell or membrane³⁹. Previous speculations on the role of various segments of these sialoglycoproteins for membrane organisation and function for the mature red cell membrane thus do not seem justified²². These cell surface molecules may be more important during earlier stages of differentiation. A genetic event involving cells committed to the erythrocytic series is expected to result in abnormal erythrocytes exclusively. We do not know when glyophorins are synthesised first and whether their synthesis is restricted to cells of the erythrocytic series. Possibly even in variant cells these proteins are made during the early phases of haematopoiesis, but are lost during later stages of differentiation. It is certain, however, that the absence of glyophorins alone does not describe all the alterations of the membrane which do occur in these variant cells and which involve other proteins and/or lipid components of the membrane¹¹.

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letters to nature

Revised estimate of gravitational radiation from Crab and Vela pulsars

PROGRESS in the development of ultra-high-Q dielectric crystals (Bagdasarov *et al.* unpublished data) has caused experimenters in the field of general relativity to look for rotation-induced gravitational radiation from pulsars (D. H. Douglass, unpublished data). If one could control the frequency of oscillation of a high-Q crystal, keeping it in phase with the electromagnetic signals observed from a pulsar, one might hope to absorb a measurable amount of (quadrupole) gravitational radiation at twice the pulsar frequency. Press and Thorne¹ in 1972 estimated that the gravitational waves from the Crab pulsar would produce a dimensionless strain in a detector on Earth of $h \sim 10^{-26}$ to 10^{-28} , and that other pulsars would be several orders of magnitude fainter. Additional observational data, and progress in pulsar models during the past five years, make a new estimate desirable. I report here that the amplitude of the gravitational waves from the Crab pulsar (PSR0531+21) is likely to be within 2 orders of magnitude of 10^{-27} , but that the Vela pulsar (PSR0833-45) is likely to produce waves of amplitude a factor 10 to 100 larger.

The waves are produced by the rotation of mass asymmetries in the neutron star. Of all conceivable mass asymmetries, conventional pulsar theory^{2,3} points to one as the most likely

to dominate the radiation: the neutron star must be rotationally flattened with oblateness $\varepsilon_0 \equiv (\text{equatorial radius} - \text{polar radius})/(\text{mean radius})$. If the star were to rotate about its polar axis of symmetry, it would not radiate gravitationally. But, the observed 'restless' behaviour of the Crab and Vela rotation periods P (refs 2, 4) makes it likely⁵⁻⁸ that the rotation axis and the symmetry axis are misaligned by a small angle θ_w . Such a misalignment would produce a 'Chandler wobble' in the star's rotation⁶, and the wobble-induced strains would produce microquakes that provide an attractive and successful explanation^{5,7} for the observed 'restlessness'. The misalignment would also cause a fraction $\varepsilon \equiv \varepsilon_0 \theta_w$ (for small θ_w) of the star's moment of inertia I to radiate as a time-changing quadrupole moment. The resulting luminosity in gravitational quadrupole radiation would be¹

$$L_{\text{GW}} = \frac{32G}{5c^5} \varepsilon^2 I^2 \left(\frac{2\pi}{P} \right)^6 \simeq 5 \times 10^{32} \text{ erg s}^{-1} \left(\frac{\varepsilon}{2 \times 10^{-6}} \right)^2 \left(\frac{I}{4 \times 10^{44} \text{ g cm}^2} \right)^2 \left(\frac{P}{0.033 \text{ s}} \right)^{-6}$$

Of the crucial parameters, P is determined by radio and optical observations⁴ to great precision. The distances to the pulsars are less well known, but the association with supernova remnants yields distance estimates of 2,000 pc and 500 pc for the Crab and Vela respectively, good to within about 25%^{1,9}. Neutron-star moments of inertia depend both upon the assumed mass of the star and upon the equation of state of matter at high densities. But because the stellar radius tends to decrease as mass increases, I is somewhat buffered; early equations of state² gave $7 \times 10^{43} \lesssim I \lesssim 7 \times 10^{44} \text{ g cm}^2$, but more recent work¹⁰ suggests $3 \times 10^{44} \lesssim I \lesssim 3 \times 10^{45} \text{ g cm}^2$. Within this range, the original Pines and Shaham⁵ 'crustquake' explanation of the Crab pulsar glitches agrees with the spindown power output ($I\dot{\Omega}$) required to drive the nebula^{2,11} and implies $I \sim 4 \times 10^{44} \text{ g cm}^2$ (though Pandharipande, Pines and Smith (PPS) for their 1.33 $M_\odot$ Crab model¹⁰ find $I \sim 2 \times 10^{45} \text{ g cm}^2$). The Vela pulsar, which has experienced at least three 'giant' speedups within the past 8 yr (refs 4, 12) is modelled as a more massive, solid-core object¹⁰ with the moment of inertia¹⁰ $I \sim 2$ to $3 \times 10^{45} \text{ g cm}^2$.

The oblateness of the star, ε_0 , is predicted by standard starquake theory and by calculations of the critical strain which the crust (for the Crab) or the core (for Vela) can withstand before

Table 1 Estimated pulsar gravitational radiation

	Standard Crab	PPS ¹⁰ Crab	Vela
$P(\text{s})$	0.033	0.033	0.089
$\omega_{\text{GW}}(\text{s}^{-1})$	380	380	140
$R(\text{pc})$	2,000	2,000	500
$I(\text{g cm}^2)$	4×10^{44}	2×10^{45}	2×10^{45}
ε_0	2×10^{-4}	4×10^{-4}	3×10^{-3}
θ_w	10^{-2}	10^{-2}	10^{-2}
$L_{\text{GW}}(\text{erg s}^{-1})$	5×10^{32}	4×10^{34}	1×10^{34}
max	1×10^{33}	1×10^{37}	2×10^{37}
min	3×10^{29}	8×10^{31}	9×10^{30}
$\mathcal{F}(\text{erg cm}^{-2} \text{ s}^{-1})$	1×10^{-12}	9×10^{-11}	4×10^{-10}
max	4×10^{-10}	4×10^{-8}	1×10^{-6}
min	4×10^{-16}	1×10^{-13}	2×10^{-13}
h	1×10^{-27}	9×10^{-27}	5×10^{-26}
max	2×10^{-26}	2×10^{-25}	3×10^{-24}
min	2×10^{-29}	3×10^{-28}	1×10^{-27}

fracturing. The resulting values are, for the Crab^{2,5,8}, $\varepsilon_o = (1-2) \times 10^{-4}$ (though the PPS $1.33 M_\odot$ model¹⁰ has $\varepsilon_o \simeq 3.5 \times 10^{-4}$), and for Vela^{8,10,13}, $\varepsilon_o \sim 10^{-2}$ to 10^{-3} . The fraction of this oblateness which is effective in producing gravitational radiation is θ_w , the wobble angle. The microquake theories suggest that θ_w is limited by starquakes to values of the order of 0.1 radians (refs 5, 7, 8); however, the lack of observed wobble⁴ (and in particular, the constancy and sharpness of the optical Crab light curve) suggests that 0.1 be taken as an extreme upper bound, and that θ_w probably lies between 10^{-3} and 10^{-2} radians. The wobble question is the most uncertain part of this analysis (see ref. 6 for comments and references).

The most probable values for the vital parameters in determining the gravitational luminosity are summarised in Table 1, for the standard Crab model, the PPS¹⁰ (stiff equation of state) $1.33 M_\odot$ Crab, and for Vela. The resulting gravitational wave luminosity L_{GW} , the energy flux at Earth $\mathcal{F} \equiv L_{GW}/4\pi R^2$, and the wave amplitude (strain, or metric perturbation)¹⁴ $h \equiv (16\pi G \mathcal{F}/c^3 \omega^2 \tau)^{1/2}$, are given together with their probable ranges using the parameter estimates described above. The errors have been added coherently, not by quadrature, so the range covered is rather large; most of the uncertainty comes from the uncertainty in θ_w .

It is possible, of course, that something is radically wrong with the above assumptions. On the other hand, if internal toroidal magnetic fields exist with $B \gtrsim 10^{15} \text{ G}$ (refs 3, 6), they could produce an oblateness $\varepsilon_o \sim 10^{-35} (B/1 \text{ G})^2$ comparable with or larger than the fluid oblateness. If the protons in the neutron star form a type-2 superconductor^{15,16} with critical field $H_{c1} - (4 \text{ to } 8) \times 10^{14} \text{ G}$, then in the low-flux-density limit ($B \ll H_{c1}$) $\varepsilon_o \sim 10^{-35} B H_{c1}$ and so smaller internal fields may begin to cause a significant oblateness. In both cases, the internal fields may tend to align themselves perpendicular to the spin axis of the star¹⁶ (effectively $\theta_w = 90^\circ$) and would thus be maximally efficient in producing gravitational radiation. An internal field of 10^{15} G would make a wave of amplitude $h \sim 5 \times 10^{-27}$ from the Crab's distance; a field of 10^{12} G in a type-2 superconductor would produce $h \sim 2 \times 10^{-30}$. So it is improbable that h is much less than the minimum estimates (2×10^{-29} for the Crab, and 1×10^{-27} for Vela) which starquake theory suggests.

The wave amplitude h could, however, be larger. Mountains or other local inhomogeneities in the crust or core could conceivably produce a net nonaxisymmetric oblateness of the same order of magnitude as the materials' shearing strengths^{2,10}, several orders of magnitude larger than the starquake models predict. An extreme upper limit on L_{GW} can be set by requiring that gravitational radiation account for the entire observed slowdown of the pulsars. That limit yields⁴ for the Crab, $L_{GW} \lesssim 2 \times 10^{38} \text{ erg s}^{-1}$, $\mathcal{F} \lesssim 7 \times 10^{-7} \text{ erg cm}^{-2} \text{ s}^{-1}$, $h \lesssim 8 \times 10^{-25}$ and for Vela, $L_{GW} \lesssim 2 \times 10^{37} \text{ erg s}^{-1}$, $\mathcal{F} \lesssim 1 \times 10^{-6} \text{ erg cm}^{-2} \text{ s}^{-1}$, $h \lesssim 3 \times 10^{-24}$, which coincidentally agrees with the upper-bound estimate for Vela on the basis of maximum credible oblateness (Table 1).

To detect the quadrupole radiation at frequencies ω_{GW} of 380 s^{-1} for the Crab and 140 s^{-1} for Vela, some workers envision using large monocrystals, probably of sapphire or silicon (Bagdasarov *et al.*, and Douglass, unpublished data). For crystals of effective length l and effective mass m , the change in amplitude of oscillation due to absorption of gravitational waves in phase with the crystal's oscillation during a measurement time τ is (assuming $\tau \ll \tau^*$, the damping time):

$$\Delta X_{GW} = \tau \omega h l / 2$$

Brownian motion at temperature T in a crystal with damping time τ^* ($\tau^* \equiv 2Q/\omega$) produces an amplitude change

$$\langle \Delta X_{\text{Brwn}}^2 \rangle^{1/2} = (2\tau k T / m \omega^2 \tau^*)^{1/2}$$

where k is Boltzmann's constant.

For a reasonable signal-to-noise ratio¹⁷ of 10, the minimum detectable wave has amplitude

$$h \gtrsim 10 \left[\frac{2kT}{mQ\omega^3 l^2 \tau} \right]^{1/2} \simeq 7 \times 10^{-28} \left(\frac{T}{10^{-3} \text{ K}} \right)^{1/2} \cdot \left(\frac{10^6 \text{ g}}{m} \right)^{1/2} \cdot \left(\frac{10^{14}}{Q} \right)^{1/2} \cdot \left(\frac{380 \text{ s}^{-1}}{\omega_{GW}} \right)^{3/2} \cdot \left(\frac{10^2 \text{ cm}}{l} \right) \cdot \left(\frac{10^7 \text{ s}}{\tau} \right)^{1/2}$$

It is straightforward to verify that detection of this signal would not require a 'quantum non-demolition sensor'^{17,18}, though the construction of the required sensor would be a nontrivial task. The minimum detectable wave amplitude for Vela is a factor of 4 higher, due to its lower frequency. The values of T , m , Q , l , and τ assumed above are not unreasonable goals for the next 5–10 yr of experimental effort¹⁹. It thus seems that, if the Crab or Vela pulsars are as strong as the best estimates indicate, they may be borderline-detectable by gravitational astronomers within the 1980s.

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Distance limit for a class of model γ -ray burst sources

THE observed time structures of γ -ray bursts of the type reported by Klebesadel *et al.*¹ place an upper limit $l \approx 10^9 \text{ cm}$ on the typical size of the burst source regions². If the sources were too far away and isotropically radiating then the observations would require such a high density of photons that the sources would be optically thick in the MeV region simply due to γ - γ -pair production. These considerations do not apply directly to highly beamed sources or relativistically expanding sources. I point out here that MeV photons have actually been observed in bursts, and that this means that non-relativistic sources cannot be further away than a few kpc from the Sun and therefore must be galactic.

Some of the available spectral burst data^{3–8} show a significant flux of photons in the $> 1 \text{ MeV}$ range^{3–5,8}. The 27 April 1972, event observed by Apollo 16 (ref. 3) shows at higher energies a power law spectrum with a possible line feature around 4 MeV. However, power law spectra and lines cannot escape with significant intensity from homogeneous optically thick sources, and if the lines were generated in different spatial regions that are optically thin it would seem to be difficult to get the energy into

these regions to excite MeV lines and nothing else. Another possibility would be to generate the burst in a thin shell surrounding the volume of radius l . But that does not significantly decrease the self-absorption of the source, as shown below.

In case of rapidly varying thermal radiation from an optically thick source it is not easy to construct a spectrum that is a power law at high energies when integrated over the burst duration. A cooling blackbody can account for some lower energy features of the burst spectrum⁹, but not for the high energy tail.

For most events there is insufficient information available on the time structure of the burst around 1 MeV. However, the 18 December 1972 event – although it was not observed at 1 MeV – showed no rapid spectral changes on the time scale of the fast sub-bursts⁷, and a generally increasing hardness near the beginning of the burst. These data suggest that the high energy fast time structure may not be significantly different from the lower energy time structure.

The significance of photon photon pair production in astrophysical situations has been investigated for a variety of cases. Nikishov¹⁰, and Fazio and Stecker¹¹ considered the absorption of very high energy photons by thermal photons in intergalactic space. Jelley¹² investigated that same mechanism for quasars and other radio sources. McBrean¹³, and Stecker and Tsuruta¹⁴ applied these considerations to the Crab pulsar NPO532, and Rengarajan¹⁵ considered the escape of γ rays from hot neutron star surfaces. In a more general assessment Herterich¹⁶ showed that intense X-ray sources are optically thick for γ rays above a few MeV and therefore cannot be high energy γ -ray sources.

To estimate the optical depth of a homogeneous, isotropic radiation field we have used the formulae as used earlier by Nikishov¹⁰, Jauch and Rohrlich¹⁸, and Gould and Schröder¹⁷. For simplicity we assume that all photon-photon encounters take place at $\theta = 90^\circ$ between the momentum vectors of two interacting photons. The threshold energy ϵ_{th} of an ambient photon for photon-photon pair production, interacting with a test photon of energy E , is

$$\epsilon_{th} = (2m^2c^4)/E(1 - \cos\theta) \quad (1)$$

Here mc^2 is the electron rest energy. The cross section $\sigma_{\gamma\gamma}$ has a maximum of $1.7 \times 10^{-25} \text{ cm}^2$ at $\epsilon_0 = 2\epsilon_{th}$.

For a typical source radius l and ambient photon density $n \text{ cm}^{-3}$, the optical depth for the test photon is typically

$$\tau(E) = l \int_{\epsilon_{th}}^{\infty} n(\epsilon) \sigma_{\gamma\gamma}(E, \epsilon) d\epsilon \quad (2)$$

With the observed photon number flux $F(\epsilon) \text{ cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}$ the spatial density of photons on the surface of the source is $n = (F/c)/(D^2/l^2)$ where D is the distance of the source; hence

$$D(E) = \left(\frac{\tau l c}{\int_{\epsilon_{th}}^{\infty} F(\epsilon) \sigma(E, \epsilon) d\epsilon} \right)^{1/2} \quad (3)$$

For integration we make two alternate assumptions for the spectral shape of the flux $F(\epsilon) \text{ cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}$. We consider (a) a composite spectrum⁴

$$\begin{aligned} & \frac{F^{(a)} \text{ cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}}{(\text{erg cm}^{-2} \text{ s}^{-1})} \\ &= 1.6 \times 10^4 \exp(-\epsilon/150 \text{ keV}) (\epsilon \leq 375 \text{ keV}) \\ &= 3.9 \times 10^9 (\epsilon/\text{keV})^{-2.5} (\epsilon > 375 \text{ keV}) \end{aligned}$$

and (b) for comparison we consider a pure power law

$$\frac{F^{(b)} \text{ cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}}{(\text{erg cm}^{-2} \text{ s}^{-1})} = 1.9 \times 10^8 (\epsilon/\text{keV})^{-2}$$

This is the photon number flux normalised to a burst of integrated energy flux of $1 \text{ erg cm}^{-2} \text{ s}^{-1}$. For normalisation of case (b) we

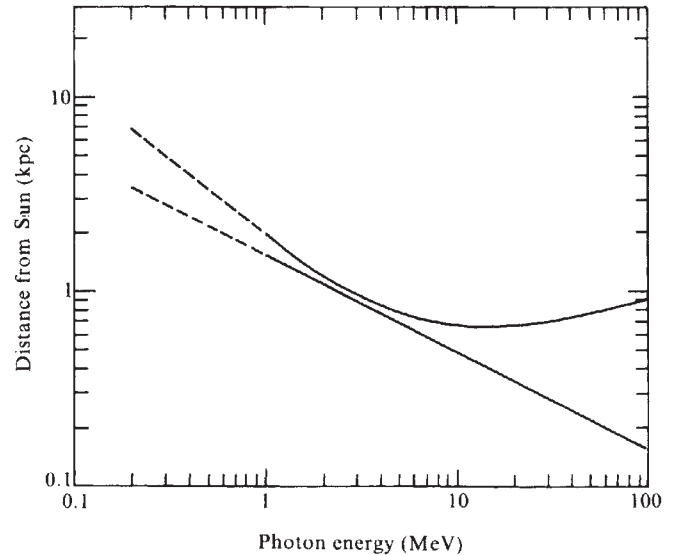


Fig. 1 Distance where source of $3 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1}$ burst is optically thick, $\tau = 1$. Homogeneous isotropically radiating burst sources are optically thick at the abscissa energy if assumed to be further away from the Sun than indicated on the ordinate. The two photon number spectra of bursts (upper curve for the composite spectrum) have been assumed without cutoffs.

have assumed that 70% of the usually quoted energy of the burst is found between 100 keV and 1,000 keV.

As an example we take a typical burst of size $10^{-4} \text{ erg cm}^{-2}$. The actual energy flux of such a burst is around $3 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1}$ with wide variations. We assume now that the optical depth of the source region should be $\tau < 1$ in order to assure the escape of the test photon. With this we arrive at Fig. 1. Since a fair number of events have been detected near 1 MeV (refs 3–5, 8), and all show deviations from an exponential spectrum, we take the $\tau = 1$ distance derived for 1 MeV for the upper limit of the distance to a $10^{-4} \text{ erg cm}^{-2}$ homogeneous isotropic burst source, which is $D \approx 2 \text{ kpc}$ according to Fig. 1. Thus this type of burst source must be galactic. The photon number flux fluctuates greatly during the burst, and the actual momentary fluxes are often significantly greater than the average fluxes. So our upper limit should be rather generous.

This distance limit was estimated for $l = 10^9 \text{ cm}$ sources which are isotropically radiating with initial photon density constant throughout the source volume and the same as on its surface. If the radiation were to emerge only from a surface around the source, but each surface element were radiating isotropically, then near the source the photon density would still be quite high, but the average angle of encounter would be less than 90° . A similar situation was considered previously¹⁹, and for our case we find that the $\tau = 1$ distance would be insignificantly larger than our upper limit. Also, if the high energy radiation of the event of 27 April 1972 were a 4.4-MeV carbon line feature with a typical time scale of about 5 s and intensity $10^{-1} \text{ s}^{-1} \text{ cm}^{-2}$, then the typical source radius would be about 10^{11} cm , and the resulting $\tau = 1$ distance would be about $3 \times 10^5 \text{ pc}$. Thus even this very extreme assumption would keep the source well within the local group of galaxies.

Within 2 kpc of the Sun there is about 1% of the galactic mass; this holds for either all matter²⁰ or the extreme population I as represented by the hydrogen disk²¹. If the bursts come from objects distributed throughout our Galaxy similar to either one of the two classes of objects then bursts of size $10^{-4} \text{ erg cm}^{-2}$ come from only about 1/100 of the Galaxy. Assuming the widely used standard candle hypothesis where the burst size is inversely proportional to the square of the source distance, the frequency of bursts of size $\geq S$ is at the same time a lower limit to the frequency of occurrences of bursts at distance $\leq D(S)$. Since the frequency of detections of $10^{-4} \text{ erg cm}^{-2}$ bursts is about three per year (refs

22, 23) the upper limit to the distance leads to an upper limit of the average intrinsic luminosity of $\approx 5 \times 10^{40}$ erg and to a lower limit of about 300 burst occurrences per year throughout our Galaxy.

This means that unless very special radiation mechanisms are at work occurrences that are unique in the history of an individual star cannot account for the majority of the bursts that are being detected. The rate of star formation in our Galaxy is at present about two to three solar masses per year²⁴ and supposedly has been constant for the last few times 10^9 yr. We assume for the sake of this argument that this means two to three stars per year. It is difficult to see how the rate of irreversible star transformations could be two orders of magnitude higher than this rate at the present epoch. Nova explosions also have been suggested as γ -ray burst sources. The rate of optical novae per year has been estimated to be about 30 to 50 in our Galaxy^{25,26}, and 25 to 30 in M31 (refs 27, 28). These numbers would be, considering the usual uncertainty of numbers in astrophysics, marginally compatible with our number of burst occurrences. However, considering the high degree of isotropy of the detected burst directions^{22,29} we feel we can also exclude novae as the major contributors to γ -ray bursts. Therefore, we suggest tentatively that the γ -ray bursts that have been detected are galactic, but are in the majority of the cases not connected with unique irreversible star transformations, and also it is unlikely that they are connected with galactic novae.

In the case of beamed sources the threshold energy (1) depends on the opening half angle $\Gamma/2$ of the beam. Using the same arguments as Stecker and Tsuruta¹⁴ we assume $\theta \approx 2^{1/2} \Gamma/2$. Assuming that 5 MeV photons have been observed^{3,5} we set $\epsilon_{th} \approx 2.5$ MeV and from this with (1) we derive $\Gamma/2 \approx 12^\circ$. With (3) we find that such a source should be optically thick ($\tau = 1$) at 5 MeV if placed further than 6 kpc from the Sun. The galactic fraction of matter within this distance is about 1/10 of the total, and the solid angle covered by the $\Gamma/2 = 12^\circ$ burst is 1/8 sr, so that in the same way as above we arrive at a minimum number of burst occurrences throughout the galaxy of $8 \times 4\pi \times 10 \times 3 \approx 3,000 \text{ yr}^{-1}$. Hypothetical beams narrower than 12° cannot be assessed in this way, because the energy where absorption can become important ($\lesssim 2 \epsilon_{th}$) is not covered by observations, yet. Thus for not too narrow beams the conclusions are essentially the same as above.

I emphasise here that the conclusions reached depend entirely on observed parameters. No specific assumption had to be made about particles or fields within the sources. Generally, particles or fields can also serve as absorbing agents so that their presence can only increase the optical depth of the source and therefore put even more severe constraints on the distance of the sources.

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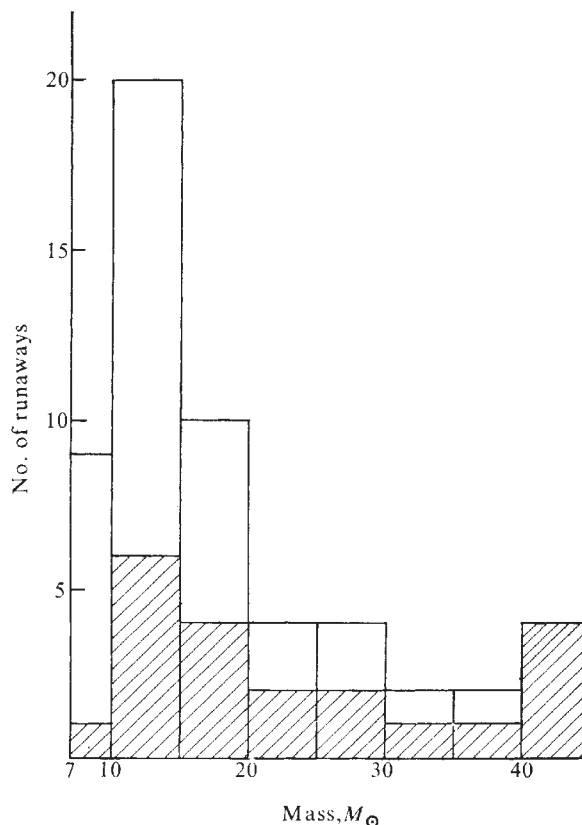
Runaway stars as witnesses to supernova explosions

THE hypothesis that single runaway stars are released from close binaries in which the companion star explodes as a carbon detonation supernova and is totally disrupted¹ is proposed here. This is contrary to the earlier belief² that at the time of the explosion the more evolved star is the less massive so that the supernova explosion which leaves behind a relativistic star core does not disrupt the binary³ but accelerates it to a velocity of a few tens of km s^{-1} (ref. 4). The binary consisting of a normal OB star and an inactive compact star could be detected as a runaway till it shows itself as an X-ray source.

From the stars that can explode as supernova, it is only the lower mass stars that burn carbon explosively⁵. Therefore if the above hypothesis is correct, lower mass runaways should be predominantly single, while the higher mass runaways should be predominantly binaries containing inactive compact stars. This is shown to be the case in Fig. 1 (based on data from ref. 6). Of the 55 runaways (peculiar velocities $\geq 40 \text{ km s}^{-1}$) listed, 29 have masses $< 15 M_\odot$ out of which only 7 (24%) are deduced to be binaries on the basis of radial velocity variations. On the other hand, out of 26 runaways with masses $\geq 15 M_\odot$, 14 (54%) are deduced to be binaries.

For a supernova explosion to take place in a close binary, the primary (the 'first' star) should start with a mass $\geq 15 M_\odot$. If the

Fig. 1 Mass distribution of runaways. The shaded area refers to the distribution of runaway binaries containing inactive compact stars. Runaways with masses $\geq 40 M_\odot$ are clubbed together.



second star is to collect the matter being transferred from the first star it should have a main sequence mass of about $4 M_{\odot}$ (ref. 3), because if the mass ratio is smaller than 0.3, mass transfer to the second star would make it thermally unstable, leading to the formation of a contact binary and large mass loss from the system⁷. If all the mass lost by the first star is collected by the second star, there must be a lower limit on the mass of a single runaway star. Assuming that the main sequence masses of the components were $15 M_{\odot}$ and $4 M_{\odot}$ and that the mass transfer left the first star as a helium star of mass $5 M_{\odot}$ whose supernova explosion disrupted the binary, the minimum mass a runaway could have is $14 M_{\odot}$. The fact that there are runaways with masses as small as $7 M_{\odot}$ (ref. 6) proves that during mass transfer phase, about 50% of the mass being transferred is lost from the system. A similar mass loss has been invoked⁸ to obtain a better quantitative agreement between theory and observations for Algol-type systems.

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Azimuthal brightness variation of Saturn's ring-A and size of particles

OBSERVATIONS of the brightness of ring A of Saturn, indicate that it is not constant in azimuth. This was discovered by Camichel¹, and later studied by me². Reitsema *et al.*³ and Lumme and Irvine⁴, have confirmed these observations. The A ring is fainter in quadrants following conjunction of the particles with the Earth–Saturn line, and brighter in quadrants preceding conjunction. No azimuthal brightness variation of this type is found for the B-ring⁵. Because these observations do not show any time dependence, the effect is stationary. We will call this, fact A. A possible explanation for it has been given by Colombo *et al.*⁸. Here we give an alternative explanation for fact A, which would be valid if the particles in ring A are large, which we will show is indeed the case, using a recent measurement¹¹ of the total mass of the rings.

This observational fact implies four conditions: (1) the light showing the variation has to come from the surface of the particles (the amplitude of this variation is around 30%); (2) the particles are in synchronous rotation; (3) rotations around the planet–particle axis are not allowed; (4) the albedo of the particles changes around them, or they are elongated and tilted with respect to the orbital radius. Note that all these conditions must be satisfied. If any were not, fact A would not apply. Condition (1) rules out the bright-cloud model of Pollack⁶ for ring A. That the particles are in synchronous rotation is not a surprise, as most of the satellites of Saturn are in such a state⁷. Condition (3) implies that collisions are not capable of turning the particles around, or that they are not important today. Finally, condition (4) presents two alternatives: an albedo change over the particles, or particles with their major axis not pointed exactly to Saturn, but inclined to it. A possible explanation of fact A based on this last possibility, has been given by Colombo *et al.*⁸, using a mechanism of travelling waves related to the one that produces the spiral arms in galaxies. Another possibility based on the first alternative (an albedo variation over the particles) implies that collisions have been important in Saturn's rings in the past, and it seems to us a much simpler explanation for fact A.

To understand this mechanism, we will use Fig. 1 which shows two particles of the rings seen from above, which are about to

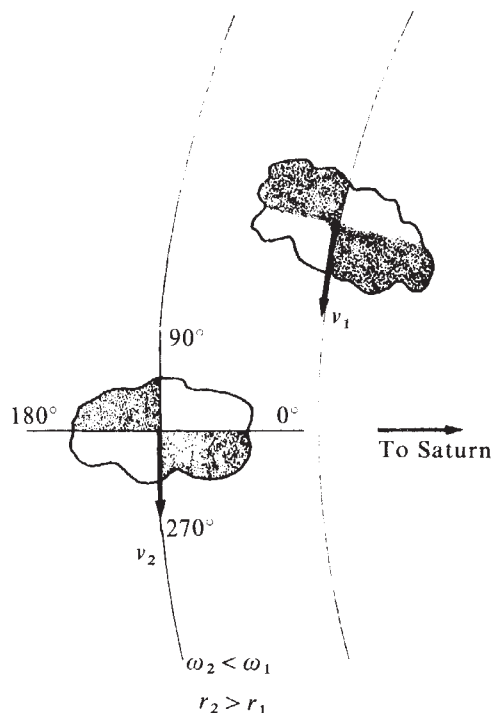


Fig. 1 Two particles about to collide in the rings. As particle 1 is nearer to the planet, it moves faster. Then collisions can occur only in the third quadrant (180° to 270°) of the first particle, or in the first quadrant (0° to 90°) of the second. There is no way in which the second and fourth quadrants of the two particles can be reached, if the particles preserve their relative radial positions. Note that the collisional regions are bright. This configuration is consistent with the azimuthal brightness variation observed in ring A.

collide. Particle 1 moves faster than particle 2, since $r_1 < r_2$, and so 1 overtakes 2. It is clear then that if we use the system of coordinates drawn on particle 2, collisions can occur only in the third quadrant ($180^\circ < \phi < 270^\circ$) of the first particle, and on the first quadrant ($0^\circ < \phi < 90^\circ$) of the second particle. There is no way in which the other quadrants can be reached. Thus we would expect some qualitative changes on the surface properties of the particles due to the effects of collisions in these two quadrants. Observationally, fact A shows that the collisions produce bright regions.

The above explanation is valid only if each particle preserves its radial positions with respect to others. Franklin and Colombo⁹ have shown that a particle at the centre of ring A is displaced by $\sim \Delta R = +147$ m in the radial direction due to perturbations by the satellites. The rate of precession of the orbits of the different particles in the rings due to the Sun and other Saturnian satellites, is a function of the radial distance to the planet. Contiguous orbits will have different rates. Secularly the radial displacements for two nearby particles will not be in phase, but their values will still be given by ΔR . So for a particle to preserve its position with respect to others, its size has to be of the same order of magnitude or larger than ΔR . If this were not the case, collisions would be allowed in all quadrants and no azimuthal variation would be produced. Thus the diameter of the particles is $D > 2\Delta R \approx 290$ m. This value again rules out the bright-cloud model of Pollack⁶ for ring A, since it requires $D \approx 10$ cm.

Additional evidence for large particles can be derived from a paper by McLaughlin and Talbot¹¹. They have obtained the total mass of the rings, from the observed secular apsidal and nodal rates of the inner satellites. They found $M_{\text{rings}} = (6.2 \pm 2.4) \times 10^{-6} M_{\text{Saturn}}$, for a rotational period of Saturn of 10 h 26 min. If a period of 10 h 14 min is adopted², their value is changed only slightly to $(5.9 \pm 2.4) \times 10^{-6} M_{\text{Saturn}}$. I have pointed out previously¹⁰ that the mass of the rings and the mean particle size are linearly related, and knowing one of them

permits the evaluation of the other. I also presented a functional relationship, for mean densities of 1.0, 2.0 and 5.0 g cm⁻³. Using the later value of the mass, we obtain mean radii of the particles of $\bar{r} = 1.9, 1.0$ and 0.5 Km, for the different densities, respectively. It is hard to escape from this conclusion, because this result is based on well known determinations of the optical thickness of the rings^{5,10}, and is insensitive to the ring model adopted.

A possible stable configuration in which the particles would preserve their radial positions could be if the particles move in an orderly fashion in smaller rings, of separation $s \gtrsim D \gtrsim 2\Delta R$ (see Fig. 2). There is some evidence that this BIAS model (beads in a string) could be correct.

Jeffreys¹² had shown that a multilayer of particles rotating around a massive primary, would transfer angular momentum inward and outward, until the ring becomes one particle thick, and the particles are separated radially by a distance equal to their diameters. The transfer of angular momentum then ceases. He derived an approximated formula for the time to reach this state: $t \simeq 10^6 \text{ yr}/D \text{ (cm)}$. This time is very short even for small particles. Presumably Saturn's rings should be in this state, and thus collision should be rare between radially neighbouring particles, in agreement with condition (3).

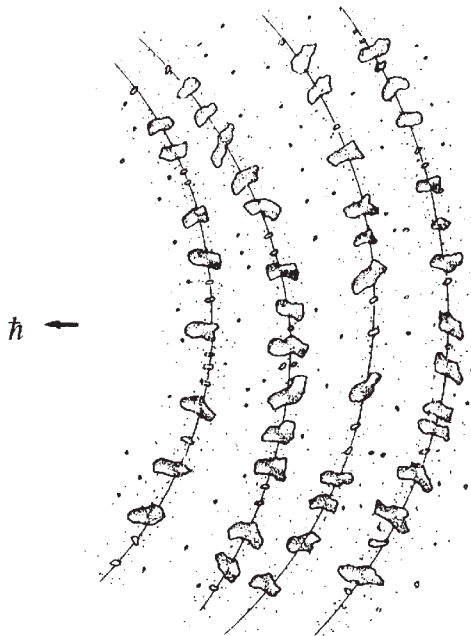


Fig. 2 BIAS configuration proposed for ring A. In this model particles move orderly in concentric sub-rings. The particles are slightly elongated, and point toward Saturn maintaining synchronous rotation. There is a fine envelope of smaller particles surrounding the rings. These would help to damp oscillations and add to the stability of the configuration. Only the large particles show an azimuthal effect.

Once the particles are in a monolayer, current theories of jet-streams^{13,17} indicate that narrow rings could be generated. In trying to prove this result, Baxter and Thompson¹⁴ have taken a population of disks in a plane, moving in keplerian orbits around a primary. Disks were taken due to mathematical simplicity. The Boltzmann-like kinetic equation for the development of the particle distribution function could be expanded as a Fokker-Planck equation for the diffusion of that function. If the elasticity of the collision were small, the diffusion coefficient would be negative, indicating that regions of enhanced density would be formed. However, a rather low value of the elasticity was found necessary for this to happen. Due to the neglect of several important physical processes, their value must be considered as a lower limit. A realistic model should include besides collisions, sliding friction between the surfaces, work done to

displace the particle from its Saturn orientation, and a possible cloud of gas and dust around the particles, that could damp all oscillations. For example, the recently discovered linear markings on the martian satellite Phobos¹⁵, could be interpreted as evidence of a sliding collision with another celestial body. From the depth of these markings it is clear that sliding friction could have been rather substantial. Incidentally, the apparition of narrow rings of enhanced density was also found for the case of accretion disks surrounding black holes¹⁶.

Alfven¹⁷ has considered the case of several apples inside a spacecraft, rotating around a planet. He found that the apples would be found neatly located at the centre of the spacecraft, in a line parallel to the orbit. In this case the focusing is produced by the walls of the spacecraft. Particles in Saturn's rings are not inside a spacecraft, but the surrounding particles could conceivably produce the same effect.

Perhaps the best evidence in favour of a BIAS model is not theoretical but observational. Such evidence lies with the discovery of narrow rings around Uranus^{18,19}, situated $\sim 40,000$ km from the planet. At the distance of Uranus, the size of the occulted star was around 4 km, so a large resolution was not possible. But from the duration of some of the events (~ 0.7 s) the conclusion is that these rings are very narrow, and cannot be wider than a few kilometres^{18,19}. The particles composing this ring have then to be smaller than a few kilometres. Note the coincidence of this value with that for the particles in Saturn's rings. This is the best evidence that narrow rings are formed in the Solar System, and could have been formed in Saturn's rings as suggested by fact A.

In conclusion, large particles for the rings have several advantages: first, they explain fact A if particles are in a BIAS configuration; second, they explain the shift in position of the outer boundary of ring B, found by Franklin *et al.*⁹; third, it is the only way of obtaining a large mass for the rings¹⁰, as found by McLaughlin and Talbot¹¹.

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Observations of strong wind shear using pulse compression radar

THE occurrence of layers of strong wind shear is interesting because of their relationship to turbulence and because of their effect on aircraft, rockets and missiles crossing them. The most extensive source of data on wind shear is from ascending balloon-borne targets but these provide individual profiles of uncertain representativeness and often lack vertical resolution. Measurements from aircraft provide more detailed data. Other sources of high resolution data being used increasingly are

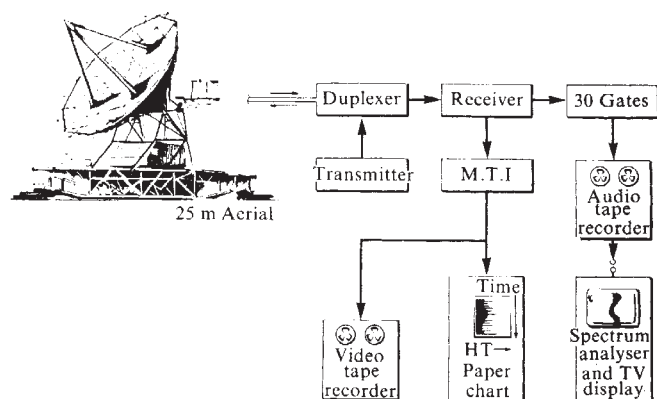


Fig. 1 Forms of data output from the Defford radar.

remote probing techniques capable of measuring the Doppler shift from windborne natural targets, such as, cloud or dust particles and inhomogeneities of temperature and humidity. Although most of these techniques have been developed for use at short range in the atmospheric boundary layer, microwave pulsed Doppler radar is one technique by which a ground-based installation can sometimes obtain wind measurements to quite high altitudes¹. Normally the spatial resolution of such radars is on the order of hundreds of metres. Here we report some observations of very strong shear made using a pulsed Doppler radar specially designed to achieve high spatial resolution.

The 25 m diameter aerial at Defford² has been coupled to a 10.7 cm wavelength radar (an FPS-18 on loan from the National Center for Atmospheric Research) redesigned to give a fine range resolution together with full Doppler measuring facilities. At present the peak power is limited to 320 kW. It has been modified to transmit frequency-modulated pulses of length 600 m. After reflection from targets these pulses are received, compressed and range-gated to give a resolution in range of 30 m. This is comparable with the beam-width resolution at a range of about 5 km.

The high gain of the aerial (53.2 dB), the low receiver noise temperature (170 K) and compression ratio (15 dB) produce a high sensitivity. The minimum detectable reflectivity for an extended target is $1.2 \times 10^{-15} \text{ m}^2 \text{ per m}^3$ at a range of 5 km. Two phase sensitive detector outputs from the receiver give the in phase and quadrature components of the Doppler frequency. They enable velocities to be measured unambiguously over the range $\pm 25 \text{ ms}^{-1}$. As large returns from the ground, received in the aerial side-lobes, tend to mask the wanted targets, a range gated MTI (moving target indicator) has been built to filter out the stationary targets. This device has 256 gates, each 30 m wide, covering a total range of 7.5 km which can be set anywhere between 0 and 100 km.

The methods of recording the data are shown in Fig. 1. The output of the MTI is displayed on a rapidly processed photo-

graphic strip chart to provide an intensity modulated time-range record of target reflectivity in real time. Doppler information from 30 selected range gates covering a total range of 900 m are also recorded on audio tape. This can be replayed rapidly and frequency spectrum analysed to give an RV (range-velocity) display on a colour television. Different colours represent different levels of echo intensity in each element of the RV matrix. If other batches of 30 range gates need to be processed similarly they are extracted from a video recording of the raw radar data.

Radar observations described here were obtained between 1446 and 1454 GMT on 30 March 1977. A balloon-borne radiosonde released during this period from the radar site showed a strongly sheared layer of strong static stability in the middle troposphere between 6.0 and 7.7 km. The Richardson number over much of this layer, evaluated over 430 m height intervals (Fig. 2), was close to 0.25. This is regarded as the critical value for the onset of Kelvin Helmholtz (KH) shearing instability³.

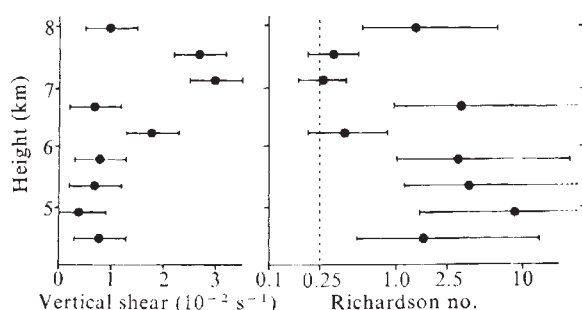
The radar beam was fixed throughout the period of the observations, looking at an elevation angle of 60° into the direction (330°) of both the wind and the wind shear vector over the strongly sheared layer. The 60° elevation angle was a compromise which enabled the radar to detect a major component of the horizontal wind at the same time as obtaining a nearly vertical profile of this component. Figure 3a is a print of the rapid processor time-range display. The ordinate is labelled in terms of true height allowing for the 60° elevation of the radar beam. Figure 3a shows the pattern of echo received mainly from small ice particles at the top of a cirrus cloud deck. Before 1440 the cloud top consisted of gently and uniformly inclined streamers of ice particles falling at less than 1 ms^{-1} . Figure 3a shows that, for a time after 1440, these streamers were distorted in a wave-like manner in the strongly sheared layer between 6.0 and 7.7 km. The crests of the waves were displaced to the right in the upper half of the layer and the troughs were displaced to the left in the lower half of the layer so as to be situated almost directly beneath the crests in the upper part of the layer. This is consistent with the pattern of air motion associated with large-amplitude KH billows⁴. This paper illustrates the capability of the radar to reveal the regions of strong wind shear which were associated with these billows.

Two examples of the format of the basic high-resolution range-velocity data are shown in Fig. 4. Velocity-height matrices such as these were obtained at 3 s intervals. Each element in the matrix is 26 m in height (30 m in slant range) by 0.625 ms^{-1} in radial velocity. As the likely fall speed of the ice particle tracers and the maximum vertical air velocity inferred from the slope of the streamlines of Fig. 3a was small ($< 1 \text{ ms}^{-1}$) compared with the horizontal wind ($> 20 \text{ ms}^{-1}$), the horizontal wind velocity can, to a first approximation, be taken as twice the indicated radial Doppler velocity component. Using such data, we have derived a time height diagram of the inferred horizontal wind speed for the top half of the disturbance (Fig. 3b). We have also derived the pattern of vertical wind shear over 52 m height intervals (Fig. 3c). Although neglect of vertical air motions does create errors in wind shear of up to $\pm 30\%$ in places, a corrected pattern of wind shear which has been derived, whilst different from Fig. 3c in detail, does not dramatically alter the overall pattern of shear.

At 1436 GMT, before the billows developed, the Doppler measurements had shown that the shear over the 400 m height interval between 6.75 and 7.15 km was uniform at $2.5 \times 10^{-2} \text{ s}^{-1}$. At times, however, during the period depicted in Fig. 3 the shear within this height interval increased locally by as much as a factor of 8, when evaluated over 52 m height intervals. The resulting shear, $20 \times 10^{-2} \text{ s}^{-1}$, can be regarded as very strong for the free atmosphere. The small vertical extent and the transient nature of the regions of strong shear would make them difficult to observe by more conventional *in situ* sensing techniques.

A pulsed microwave radar technique for obtaining high resolution measurements of wind shear has been described. The

Fig. 2 Vertical profiles of wind shear and Richardson number derived over layers 430 m deep from the radiosonde released at 1450 GMT.



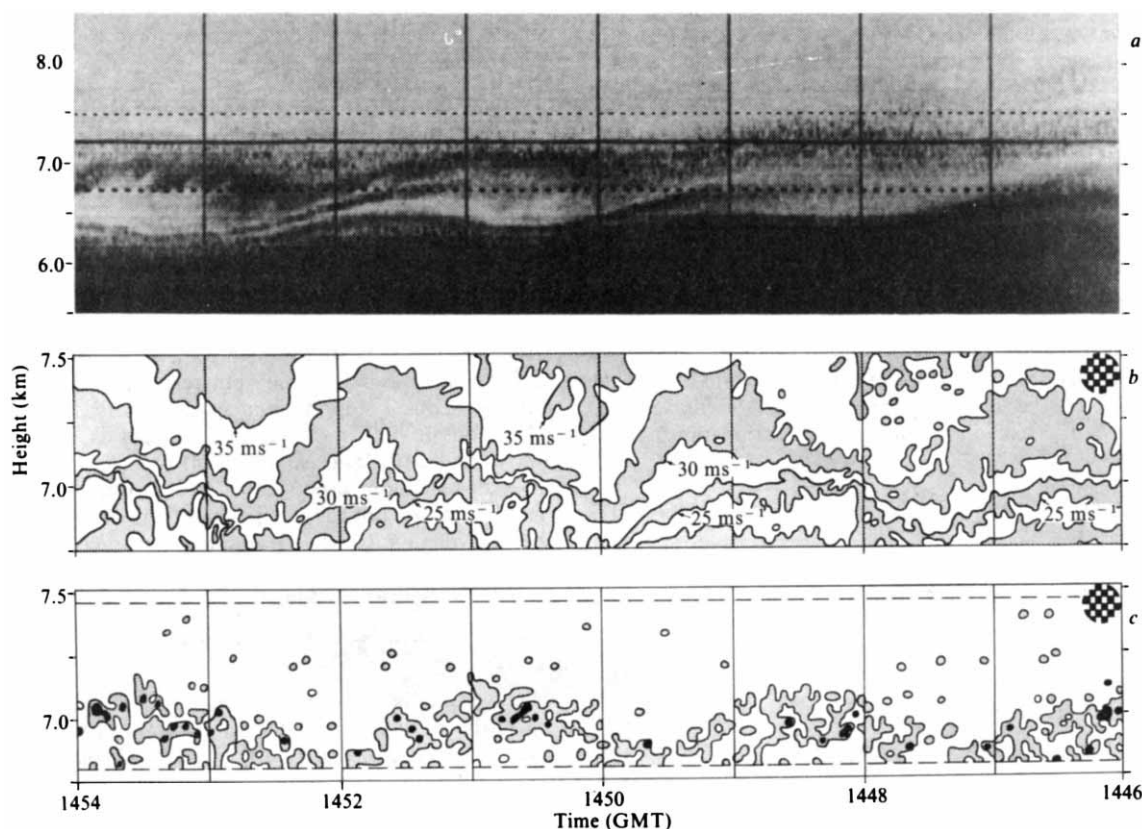


Fig. 3 Time-height record of (a) intensity of radar echo between 1446 and 1454 GMT on 30 March 1977 showing wave-like perturbations at the top of a layer of cirrus cloud. Assuming that the pattern was advecting at the speed of the wind at the middle of the perturbed layer, the wavelength would have been 3.4 km. Time-height records are also shown of (b), inferred horizontal wind velocity and (c), inferred vertical wind shear computed over layers 52 m deep. The data in (b) and (c) are for the height interval shown between dotted lines in (a): note the change in vertical scale. Isopleths of velocity in (b) are at 2.5 ms^{-1} intervals. In (c) regions of vertical wind shear exceeding $5 \times 10^{-2} \text{ s}^{-1}$ and $10 \times 10^{-2} \text{ s}^{-1}$ are dotted and black, respectively.

technique has been illustrated by means of a case study in which a vertical wind shear as strong as 0.20 s^{-1} was measured at an altitude of 7 km. The case described utilised small ice particles as tracers of the air motion. It would also work using signals back-scattered from refractive index inhomogeneities associated with humidity and/or temperature fluctuations which, with the present radar, are detectable most of the time in the boundary layer and for some of the time in the upper atmosphere, especially where there is strong shear and turbulence. As the radar operates at a wavelength virtually unattenuated by rain and water vapour it has an all-weather capability. High spatial resolution and an all-weather capability can also be achieved using frequency modulated continuous wave (FMCW) Doppler

radar techniques⁵ but so far such techniques have been restricted mainly to measuring winds in the atmospheric boundary layer.

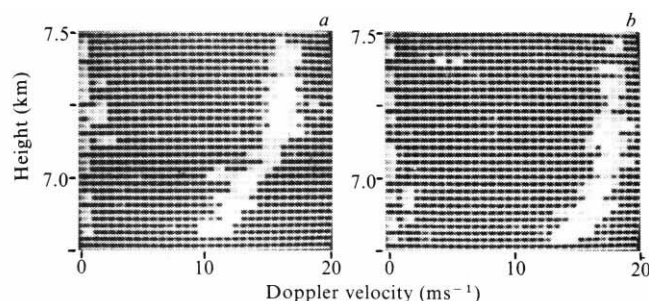
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Fig. 4 Monochrome photographs of the Doppler velocity versus height colour TV display at (a), 1449.00 and (b), 1449.57 GMT showing the region of wind shear and its change in height between the crest and trough of a billow (colours in the original display represented the intensity of the radar echo).



Distribution of helium in metal tritides

THE first generation of fusion reactors will utilise the reaction between deuterium and tritium nuclei. Various metal tritides, therefore, have potential applications for the transport, storage, purification, isotope enrichment and gettering of tritium. Although many properties of the metal tritides can be estimated¹ from the corresponding hydrides and deuterides, the effects of the radioactive triton decay into a β -particle and ^3He atom with a half life of 12.3 yr can only be determined by direct observation. Considering the expected² insolubility of inert ^3He atoms in metal lattices, it is surprising that many

metal tritides^{1,3,4} contain ³He/T atom ratios in excess of 0.10. The physical state of the retained ³He in these tritides is a very interesting and unresolved problem. Weaver and Camp^{5,6} suggest the ³He atoms are trapped in the octahedral interstitial sites for tritides with fluorite crystal structures while Bowman, *et al.*¹ believe the ³He atoms primarily reside in microscopic gas bubbles with dimensions < 1,000 Å. Here we describe nuclear magnetic resonance (NMR) studies of the relaxation times for ³He nuclei in LiT, TiT_{1.9}, and UT₃, which are representative of ionic, transition metal, and actinide tritides. These NMR measurements support the ³He bubble concept and further indicate an increase in the mean bubble dimensions as the samples age.

The NMR relaxation times are directly related⁷ to the physical state of the resonant spins. When the nuclei are immobile on lattice sites in a solid, the relaxation times obey the relations

$$T_2^* \approx T_2 \ll T_1 \quad (1)$$

where T_2^* is the linewidth relaxation time which corresponds to the experimental linewidth, T_2 is the homogeneous spin-spin relaxation time, and T_1 is the spin-lattice relaxation time. $T_2 < 50 \mu\text{s}$ are typically observed⁷ for rigid atoms in a solid. The molecular motion in macroscopic quantities of bulk gas yields

$$T_2^* < T_2 \approx T_1. \quad (2)$$

The differences in the relaxation times for rigid and mobile spins are dramatic with T_2^* and T_2 increasing by 2–4 orders of magnitude. The measured ³He relaxation times in LiT, TiT_{1.9}, and UT₃ are summarised in Table 1. Standard pulse NMR techniques⁸ were used to determine these relaxation times. The tritides were prepared by reactions at elevated temperatures between pure metals and tritium gas. Samples were sealed in evacuated glass tubes before commencing the NMR studies. The nonexponential decays generally observed during the NMR measurements are indicative^{9,10} of a variety of localised and isolated environments for the ³He spins (that is clusters or microbubbles).

Although the absolute magnitudes of the ³He relaxation times are different for LiT, TiT_{1.9}, and UT₃, these tritides have a common obedience to

$$T_2^* < T_2 < T_1 \quad (3)$$

rather than equation (1) or (2). Furthermore, T_2 and T_1 for ³He in each tritide become significantly longer with age (as the retained ³He contents increase). This behaviour implies a remarkable similarity in the ³He distributions for materials with very different¹ physical characteristics. The observed T_2^*

reflect the relative inhomogeneous line broadening contributions arising from the bulk magnetic susceptibilities¹¹, which accounts¹⁰ for the very short T_2^* in the strongly paramagnetic UT₃.

Nuclear relaxation times for ³He atoms in gas bubbles can be represented by the phenomenological expressions

$$T_1^{-1} = T_{1B}^{-1} + T_{1W}^{-1} \quad (4)$$

$$T_2^{-1} = T_{2B}^{-1} + (\lambda/R)T_{2W}^{-1} \quad (5)$$

where T_{1B} and T_{2B} correspond to bulk gas, T_{1W} is the spin-lattice relaxation at the bubble wall, R is the average bubble radius, and T_{2W} is the spin-spin relaxation time in a surface film of depth λ (approximately 2–3 atom layers). As spin relaxation in bulk monoatomic ³He gas is very inefficient⁷, $T_{2B} \approx T_{1B} > 10^3\text{s}$ and equations (4) and (5) are dominated by the wall relaxation processes. Although the surface relaxation mechanisms are not fully understood, any surface paramagnetic species will rapidly relax ³He spins during collisions with the bubble wall. Assuming relaxation occurs only in the short ($\sim 10^{-13}\text{s}$) collision sticking time t_s , T_{1W} is predicted¹⁰ to be

$$T_{1W} = (4R/3 < v > t_s) T_{1S} \quad (6)$$

where $< v >$ is the average thermal velocity of ³He atoms and T_{1S} is the surface relaxation time. As T_{1S} should be extremely sensitive to the electronic structure and chemical composition of the host material, very different ³He T_1 values for each tritide are expected and observed in Table 1. Furthermore, equation (6) predicts T_1 becomes longer as the bubble radius R is increased. T_{2W} can also be described by a relation similar to equation (6) where $T_{2S} < T_{1S}$ because the semi-static dipolar interactions which dominate T_2 relaxation⁷ would be strongest between ³He atoms near the wall. Consequently, ³He spin relaxation in bubbles would obey $T_2 < T_1$, and T_2 , with an approximately R^2 dependence, would increase more rapidly than T_1 as the bubbles enlarge.

The ³He relaxation times in Table I for LiT, TiT_{1.9}, and UT₃ are in good qualitative agreement with growing microscopic bubbles for the helium distributions in these tritides. Unfortunately, several factors prohibit a more definitive analysis. First, a range of bubble sizes (each with its own characteristic T_1 and T_2) as described by Gruber¹² is more likely than uniform bubble dimensions and the NMR studies will correspond to an 'average' bubble dimension. Various magnetic field gradients also contribute¹⁰ to the observed ³He relaxation times and tend to magnify the R -dependence for T_2 . The T_{1S} and T_{2S} values are unknown for these materials and may also have inherent time dependences which could modify the relationships between observed ³He relaxation times and bubble dimensions. Nevertheless, the ³He relaxation times observed in LiT, TiT_{1.9}, and UT₃ have been shown to be more consistent with helium atoms in microscopic bubbles whose average dimensions increase with age than the alternative explanation^{5,6} of rigidly trapped interstitials. The absence of known T_{1S} and T_{2S} values precludes a reliable determination of the bubbles dimensions from the NMR data in Table 1. However, bubble radii in the range of 10–100 Å are reasonable estimates. High resolution electron microscopy studies are required to establish better the bubble size distribution in the metal tritides.

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Table 1 Measured ³He relaxation times in LiT, TiT_{1.9} and UT₃. The NMR resonance frequency was 20 MHz for UT₃ and 45.7 MHz for LiT and TiT_{1.9}.

Material	Age (d)	T_2^* (ms)	T_2 (ms)	T_1 (ms)
LiT	184	0.23	15	6,500
LiT	346	0.47	26	7,800
LiT	541	0.44	35	9,350
LiT	735	1.04	125	10,000
TiT _{1.9}	168	0.049	0.9	500
TiT _{1.9}	340	0.062	3.2	810
TiT _{1.9}	671	0.065	10.2	1,200
TiT _{1.9}	933	0.072	45	1,500
UT ₃	150	0.020	1.1	35
UT ₃	366	—	6.1	39.5
UT ₃	836	0.020	16.0	74
UT ₃	1108	0.024	32.3	93

The T_2 values were obtained using the Carr–Purcell–Meiboom Gill pulse sequence⁸ with a 200 μs spacing between the 180° pulses. All measurements were performed at room temperature.

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Exsolution in almandine-pyrope-grossular garnet

MINERALS of the garnet group are common in metamorphic rocks and in xenoliths derived from the Earth's mantle. They consist of multicomponent solid solutions with the general formula $X_3^{2+}Y_2^{3+}Si_3O_{12}$, where X represents Fe^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} in 8-coordination, and Y represents Al^{3+} , Fe^{3+} , Cr^{3+} in 6-coordination. Solid solution between $Fe_3Al_2Si_3O_{12}$ (almandine), $Mg_3Al_2Si_3O_{12}$ (pyrope) and $Ca_3Al_2Si_3O_{12}$ (grossular) had been generally thought to be complete at temperatures appropriate to metamorphism. This paper reports the observation of exsolution in a natural garnet of this series from a high-grade metamorphic rock. Electron microscopic techniques have been used to demonstrate the phase separation of iron-rich precipitates from a more calcic matrix.

Theoretical considerations of the approximate thermodynamic properties of pyrope-grossular solid solutions by Ganguly and Kennedy¹ suggested the possibility of exsolution below about 700 °C, but attempts to demonstrate this experimentally proved inconclusive. Experimental data on the mixing properties of almandine-grossular solid solutions indicate the probable existence of a miscibility gap in this system at temperatures as high as 900 °C (ref. 2). The two-phase region calculated from these data occurs towards almandine-rich bulk compositions. These mixing data will be published elsewhere³.

In order to verify the experimental results a natural garnet of bulk composition almandine_{0.49} pyrope_{0.21} spessartine_{0.01} grossular_{0.29}, described by Wood⁴ from a metagabbro (specimen 16) in South Harris, was investigated by scanning and transmission electron microscopy. The metagabbro has been metamorphosed in granulite facies conditions, and Wood⁴ has determined the probable equilibrium pressure and temperature at the peak of metamorphism to be 10–13 kbar and 800–860 °C. The bulk composition of the garnet from this rock lies within the experimentally indicated two-phase field. Scanning electron micrographs of this garnet etched with 5% HF solution show preferential etching which produces surfaces composed of roughly spherical-shaped particles of various sizes up to about 0.5 µm diameter. The garnet seems to be composed of a microaggregate of particles.

Transmission electron micrographs of ion-thinned samples show the garnet to be composed of many individual polyhedral particles, set in a matrix which has been more easily etched by the ion-thinning process (Fig. 1). The size of individual polyhedra is variable up to about 0.4 µm, but most are less than 0.1 µm in diameter. The larger polyhedra often appear to be distributed in zones within the garnet (Fig. 1b). Contrast features in the matrix also suggest a finer scale of precipitation. The size and distribution of exsolved polyhedra may reflect the retrograde pressure temperature history of the rock, but further coarsening of exsolution textures at low temperatures may be hindered by small diffusion coefficients for garnet.

Analytical electron microscopy at 100 kV using EMMA-4 (ref. 5) has enabled the chemistry of the polyhedra and of the matrix to be determined. By focusing the beam into a small probe it is possible to analyse individual areas of 0.1–0.2 µm diameter. The matrix and polyhedra both have garnet stoichiometry, but the matrix is richer in calcium than the adjacent polyhedra. Results of chemical analyses calculated as mole fractions of grossular component (Ca/Ca + Mg + Fe) are shown

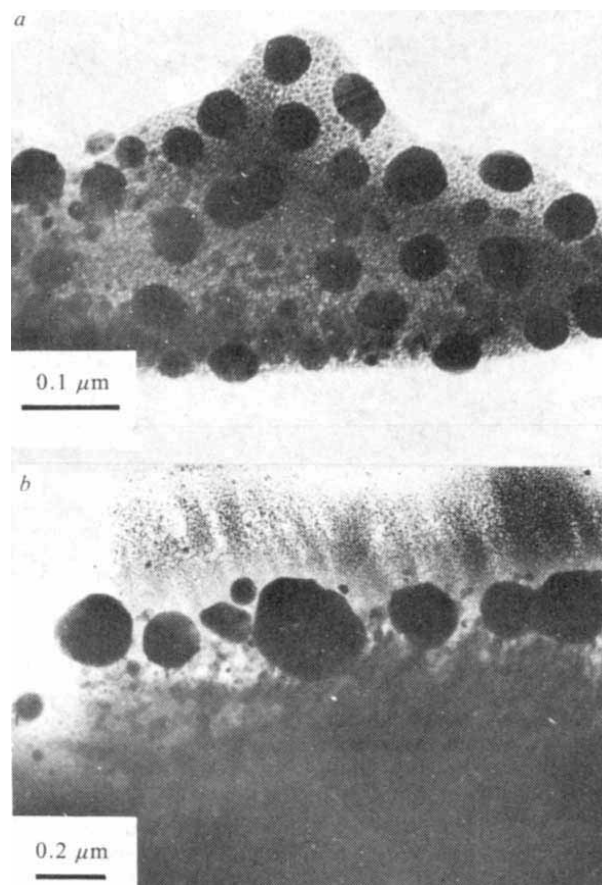


Fig. 1 Transmission electron micrographs of ion-thinned garnet, showing exsolved iron-rich garnet polyhedra. The polyhedra appear dark, partly due to diffraction contrast and partly due to thickness, as the more calcium-rich garnet matrix is preferentially etched during the ion-thinning process. Smaller precipitates are also visible in the matrix. *a*, Scale bar 0.1 µm; *b*, 0.2 µm.

in Table 1. Defocusing the electron beam to cover an area containing both exsolved polyhedra and matrix gives an analysis representative of the bulk composition, which is in good agreement with conventional microprobe results⁴.

An electron diffraction pattern from an area including many small polyhedra within the matrix is shown in Fig. 2. Dark field microscopy indicates that the regular array is from the matrix and the spotty powder rings from the randomly orientated polyhedra; both index as garnet. Cell-edges calculated from the regular array and from the powder rings differ relatively by 0.12 Å, with the regular array from the garnet of larger cell-edge. This value of 0.12 Å corresponds to a compositional difference in pyrope-almandine-grossular solid solutions of about 0.25 mole fraction of grossular component, in agreement with the chemical difference found using EMMA-4 and with the

Table 1 Garnet analyses

	Polyhedra	EMMA-4 Matrix	Bulk	Microprobe ⁴ Bulk
Ca/Ca + Mg + Fe (mol fraction)	0.22 0.20 0.19 0.21	0.37 0.32 0.40 0.35	0.28 0.29 0.29 0.26	0.29
Average	0.20	0.36	0.28	0.29

EMMA-4 analyses represent a minimum compositional separation, as with a probe size of 0.1–0.2 µm it is often not possible to analyse single polyhedra without including some matrix. Also, the matrix often contains very small precipitates. Therefore, areas suitable for analysis of mainly single phases were carefully chosen.

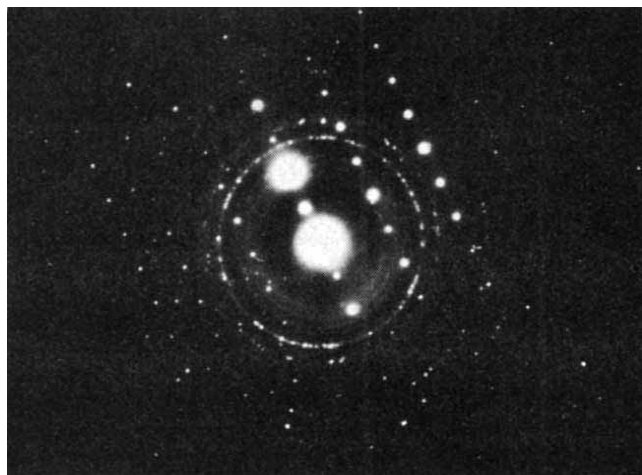


Fig. 2 An electron diffraction pattern from an area including many small polyhedra within the matrix. The regular array is from the matrix and the spotty powder rings from the randomly orientated polyhedra. Garnet cell-edges differ by 0.12 Å.

experimental mixing data. The diffraction pattern is, therefore, consistent with the interpretation that randomly-orientated garnet polyhedra have exsolved from a more calcium-rich garnet matrix.

Two distinct garnet phases produced by an exsolution mechanism are likely to be observed in almandine-pyropgrossular garnets from metabasic rocks, as garnets from this paragenesis generally have more calcic bulk compositions (up to 30 mol % grossular) than those from metapelites. Exsolution may also occur in more complex garnet solid solutions which contain the components $\text{Ca}_3\text{Cr}_2\text{Si}_3\text{O}_{12}$ (uvavovite) and $\text{Ca}_3\text{Fe}_2\text{Si}_3\text{O}_{12}$ (andradite) in addition to almandine, pyrope and grossular end-member components.

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Standards for stable isotope measurements in natural compounds

RESEARCH based on stable isotope variations in natural compounds is expanding in scientific fields such as geochemistry, hydrology, environmental studies and biochemistry. However, intercomparison of results obtained in different laboratories is often not fully reliable and therefore to improve the intercalibration of deuterium and ^{18}O measurements in natural waters, two water standards have been distributed by the International Atomic Energy Agency since 1968. The two standards, called V-SMOW (Vienna Standard Mean Ocean Water) and SLAP (Standard Light Antarctic Precipitation), were prepared, following a recommendation by an Advisory Group Meeting convened by the IAEA in 1966. Information on the two standards is given here.

V-SMOW was obtained by mixing distilled ocean water with small amounts of other waters in order to bring its isotopic composition as close as possible to that of the defined SMOW¹.

According to the control analyses made by Craig, V-SMOW has the same $^{18}\text{O}/^{16}\text{O}$ ratio as the defined SMOW, but a slightly lower D/H ratio (0.2‰). This difference in deuterium content, however, is 5 to 10 times lower than the experimental error of many laboratories.

The D/H absolute ratio² of V-SMOW was $(155.76 \pm 0.05) \times 10^{-6}$. The $^{18}\text{O}/^{16}\text{O}$ absolute ratio³ was $(2005.20 \pm 0.45) \times 10^{-6}$. No determinations were made of the $^{17}\text{O}/^{16}\text{O}$ ratio. The absolute maximum density of V-SMOW is $999.975 \text{ kg m}^{-3}$ (see ref. 4). The tritium content of V-SMOW, determined at the IAEA by direct gas counting, was $18.5 \pm 3.6 \text{ T.U.}$ on 16 September 1976 (1 T.U. (tritium unit) corresponds to 1 atom of tritium per 10^{18} atoms of hydrogen).

SLAP was obtained by melting a sample of firn collected at Plateau Station, Antarctica. Its D/H absolute ratio² was $(89.02 \pm 0.05) \times 10^{-6}$. The tritium content of SLAP was $374 \pm 9 \text{ T.U.}$ on 16 September 1976.

Two other water standards, the so-called NBS-1 and NBS-1A—formerly kept at the National Bureau of Standards, Washington DC, USA, and now at the IAEA in Vienna—were also distributed together with V-SMOW and SLAP.

In September 1976, a consultants' meeting was convened by IAEA to review the results obtained by different laboratories for the above water standards, to discuss the intercalibration

Table 1 Deuterium intercalibration results expressed in $\delta\text{D}‰$ against Vienna-SMOW

Laboratory*	NBS-1	NBS-1A	SLAP
1	−45.1	−180.3	−424.2
6	47.8	−179.6	−420.4
9	−46.4	−181.0	−423.2
11	−47.6	−182.2	−426.5
12	−47.5	−182.7	−428.5
14	−49.2	−182.9	−435.0
16	−47.2		
17	−46.3	−181.2	−424.0
18	−45.7	−182.1	−425.4
20	−47.2	−183.1	−426.1
23	−45.4	−181.2	−430.6
24	−48.5	−183.2	−427.9
26	−46.6	−180.4	−418.1
27	−44.7	−179.6	−420.2
28	−48.6	−182.0	−424.2
29	−45.1	−181.0	−421.8
30	−45.8	−183.7	−428.3
31	−46.9	−182.4	−242.4
32	−46.9	−185.7	−436.2
35	−46.1	−181.1	−423.5
36	−47.4	−181.4	†
37	†	†	−428.7
38	−48.0	−183.5	−428.2
39	−49.8	−194.2	−452.2
40	−46.5	−182.0	−425.8
41	−49.0	−182.6	−428.5
44	−48.8	−182.0	−425.5
45	−46.0	−184.0	−428.3

*Nos refer to laboratory identification included in reprints.

†Not measured, but measurements made on individual batches of Antarctic water before mixing to produce the SLAP ranged from −423 to −434 (Craig, personal communication), with an average value of −429.2 against Vienna-SMOW.

‡The SLAP value has been obtained by assuming for NBS-1 and NBS-1A the δD values of −47.6 and −183.3‰ corresponding to those reported by Craig against the defined SMOW.

of stable isotope measurements in natural samples and to receive advice on future activities. A short summary of the discussions and of the recommendations made by the consultants is given below. A more detailed report can be obtained from IAEA.

Sets of V-SMOW, SLAP, NBS-1 and NBS-1A have been distributed to 114 laboratories representing 30 countries and 2

Table 2 ^{18}O intercalibration results in $\delta^{18}\text{O}\text{‰}$ against Vienna-SMOW

Laboratory	NBS-1	NBS-1A	SLAP
1	-7.62	-24.38	-54.92
2	-7.67	-23.99	-55.19
3	-7.72	-24.09	-55.35
4	-7.81	-24.16	-55.46
5	-8.01	-24.40	-56.07
7	-8.12	-24.37	-55.73
8	-7.90	-24.43	-53.92
9	-8.01	-24.52	-56.38
10	-7.85	-23.97	-55.04
11	-7.9	-24.65	-56.5
12	-7.98	-24.59	-56.50
13	-7.88	-24.28	-55.68
14	-7.97	-24.94	-56.46
15	-7.74		-55.0
16		-24.16	
17		-24.31	
18	-7.92	-24.36	-55.40
19	-7.77	-24.17	-55.17
20	-7.93	-24.18	-54.84
21	-8.09	-24.63	-56.09
22	-7.3	-23.45	-54.9
24	-7.76	-24.36	-55.02
25	-7.83	-23.90	-54.74
26	-7.94	-24.40	-55.49
27	-7.96	-24.62	-56.14
28	-7.90	-24.74	-56.32
29	-7.86	-24.28	-55.48
30	-7.89	-24.44	-55.75
31	-7.77	-24.08	-55.05
32	-7.86	-24.32	-55.57
33	-7.87	-24.28	-55.44
34	-8.04	-23.80	-55.75
36		-24.27	
37	-7.90	-24.26	-55.44
39	-7.86	-24.22	-55.20
40	-8.01	-24.38	
42	-7.92	-24.16	-54.53
43	-7.6	-24.5	-49.2
44	-8.05	-23.87	-54.91
45	-7.92	-24.10	-55.25

international organisations. The results submitted up to September 1976 are listed in Tables 1 and 2. The agreement among the different laboratories is reasonably good, and improves significantly by eliminating the results deviating from the average of more than two standard deviations (Table 3).

If fixed δ -values are assigned to SLAP and the δ values of NBS-1 and NBS-1A are normalised accordingly, the agreement of the results should improve. In fact, the measurement technique based on two standards should produce, in principle, results which compare better between different laboratories. In this case, however, the improvement is significant only for the δD value of NBS-1A, as shown by the decrease of the standard deviation.

To resolve confusion due to the expression of results from different laboratories in non-corresponding scales, it is recommended that all future results be expressed as δ -values relative to V-SMOW. Coherence between δ -values reported by different laboratories can be improved by adopting common δ -values for a second water reference standard with respect to which the ^{18}O and deuterium δ -scales should be normalised. It is recommended, for this purpose, to adopt the water SLAP distributed by the Agency. The recommended δ -values for SLAP relative to V-SMOW are:

$$\delta^{18}\text{O} (\text{SLAP}) = -55.5\text{‰}$$

$$\delta\text{D} (\text{SLAP}) = -428\text{‰}$$

These δ -values for SLAP were selected using data in Tables 1 and 2.

The adoption of V-SMOW as zero of the δ -scales and of prefixed values for SLAP corresponds in principle to a modification of the δ -scale which becomes:

$$\delta = (R_{\text{sample}} - R_{\text{V-SMOW}}) / R_{\text{V-SMOW}} \delta_{\text{SLAP}} \times [(R_{\text{SLAP}} - R_{\text{V-SMOW}}) / R_{\text{V-SMOW}}] \quad (1)$$

where $R = ^{18}\text{O}/^{16}\text{O}$ or D/H and δ_{SLAP} is the value adopted for ^{18}O or deuterium content of SLAP with respect to V-SMOW. The classical definition of δ as the relative difference (generally reported in ‰) of the isotopic ratio with respect to SMOW is:

$$\delta = (R_{\text{sample}} - R_{\text{SMOW}}) / R_{\text{SMOW}} \quad (2)$$

The two δ -scales coincide if $R_{\text{SMOW}} = R_{\text{V-SMOW}}$ and if the adopted δ -values for SLAP correspond to the true ones as defined by equation (2). These two conditions seem to be fairly well fulfilled and therefore in practice equations (1) and (2) coincide. In addition, errors of δ which can be introduced by an incorrect assessment of multiplicative correction factors automatically cancel from equation (1).

Therefore, the advantages of significantly improving the correct comparison of results obtained in different laboratories by the adoption of recommended δ -values for SLAP far outweigh the consequences of the implications discussed above.

The need for a third reference water sample with isotopic composition approximately midway between V-SMOW and SLAP was also recognised. This sample has been called GISP (Greenland Ice Sheet Precipitation). The IAEA will soon start distributing it, with the aim of calibration on the V-SMOW/SLAP scale.

It was agreed that most of the stock of V-SMOW, SLAP and GISP should be stored in sealed flasks of approximately 10 l each. Some of the flasks should be kept at the US National Bureau of Standards, in Washington, and the rest at the IAEA in Vienna.

About 10 l of each standard should be stored in sealed pyrex vials of about 25 ml for distribution by IAEA. In principle, V-SMOW, SLAP and GISP should be distributed upon request only, but laboratories are encouraged to make a precise calibration of their working standards on the V-SMOW/SLAP scale about once every three years.

The establishment of other stable isotope standards is required in order to improve the intercomparison of results obtained in different laboratories in natural substances other

Table 3 Intercalibration average values and standard deviations

Sample		Deuterium			^{18}O		
		δ	σ	n	δ	σ	n
NBS-1	1.	-47.0	1.4	27	-7.87	0.16	37
	2.	-47.0	1.4	27	-7.88	0.13	36
	3.	-47.1	1.2	26	-7.90	0.18	36
	4.	-47.1	1.2	26	-7.89	0.12	34
NBS-1A	1.	-182.6	2.8	26	-24.28	0.28	39
	2.	-182.2	1.5	25	-24.29	0.23	37
	3.	-182.9	1.1	26	-24.39	0.62	35
	4.	-183.2	0.7	24	-24.29	0.25	34
SLAP	1.	-427.1	6.6	26	-55.27	1.20	36
	2.	-426.1	4.2	25	-55.45	0.16	35

1. All results included as reported in Tables 1 and 2.
2. Excluding those deviating by more than 2σ from the average of 1.
3. Results normalised with respect to SLAP, assuming for the latter $\delta\text{D} = -428$ and $\delta^{18}\text{O} = -55.5$. All results included.
4. Excluding the results deviating by more than 2σ from the average of 3.

Table 4 Proposed new stable isotope standards

Compound	Elements for which standard can be used	Remarks (and workers willing to investigate the possibility of procuring standards)
CaCO ₃	C, O	Calcite as close as possible to PBD. I. Friedman (Denver, Colorado) and J. O'Neil (Menlo Park, California).
CaCO ₃	C, O	Carbonatite. H. Friedrichsen (Tübingen, FRG).
CO ₂	C, O	Two different gas samples for mass spectrometer calibration. T. B. Coplen (Reston, Virginia).
C ₂₇ H ₁₁₀ O ₆	H, C, O	Tristearin for studies of organic compounds.
K ₂ Al ₄ (Si ₆ Al ₂ O ₂₀)(OH, F) ₄	H, O	Muscovite. I. Friedman and J. O'Neil.
K ₂ (Mg, Fe) ₆ (Si ₆ Al ₂ O ₂₀)(OH, F) ₄	H, O	Biotite. I. Friedman and J. O'Neil.
BaSO ₄	O, S	Obtained from sea water. Y. Horibe (Tokyo, Japan).
PbS or ZnS	S	Natural galena or sphalerite with $\delta^{34}\text{S}$ in the range -20 to -30% . I. Friedman and J. O'Neil.
S	S	Sulphur derived from natural gas, depleted in ^{34}S . E. Roth (Saclay, France).
SO ₂	S	Two different gas samples for mass spectrometer calibration. J. O'Neil and I. L. Barnes (Washington D.C.).
(NH ₄) ₂ SO ₄	N	Two samples differing by about 20‰. E. Salati (Piracicaba, São Paulo, Brazil).
KNO ₃	N	I. Friedman and J. O'Neil.

The so-called PBD standard consisted of a CaCO₃ obtained from a belemnite of Peedee formation of South Carolina. Its ^{13}C and ^{18}O contents are supposed to be close to the average contents of marine limestone. The PDB standard was first adopted in the early 1950s as reference in palaeotemperature studies.

than water. The proposed new reference samples, which will be kept at both IAEA and US National Bureau of Standards, are listed in Table 4. They will supplement the standards now being distributed by the US National Bureau of Standards which, in some cases, are insufficient in amount for a long-term worldwide distribution (as, for instance, the samples of nitrogen (NBS-14) and of SO₂ (NBS-120A), I. L. Barnes, personal communication).

SLAP was prepared by H. Craig, University of California at San Diego; GISP was provided by W. Dansgaard, University of Copenhagen. A list of laboratories participating in the stable isotope intercalibration is contained in the reprints of this article.

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Silica oxygen isotopes in diatoms: a 20,000 yr record in deep-sea sediments

PALAEOTEMPERATURE determinations based on oxygen isotope composition of planktonic foraminifera are well established for Quaternary and Tertiary deep-sea sediments¹⁻⁴. Wide areas of the ocean floor, however, are lacking suitable calcareous fossils due to dissolution or unfavourable ecological conditions. Here we show that isotopic analyses of diatom frustules can adequately substitute for the analyses of calcareous microfossils. To test the efficiency of diatoms as an isotopic palaeothermometer^{5,6}, we compare the oxygen isotope records of diatoms and of planktonic foraminifera in a box-core from the eastern equatorial Pacific (PLDS 72, latitude 01°08'N; longitude 109°15'6''W; water depth 3,626 m). This core was among other box-cores raised during the S.I.O. Pleiades expedition between May and September 1976, in an effort to obtain undisturbed samples of the uppermost deposits in this fertile area of pelagic sedimentation.

Isotope analyses of the planktonic foraminifera followed

standard procedures⁷ and the reproducibility of the oxygen isotope values is about $\pm 0.1\%$. All box-cores were routinely assessed isotopically at Scripps Institution of Oceanography to establish stratigraphic continuity.

The preparation of biogenous silica for isotopic analysis requires considerably more effort than that of calcareous materials. The diatoms are separated from carbonate, radiolarians and detritals by HCl treatment, ultrasonication, sieving (fraction larger than 22 μm retained) and differential settling. The process further requires a complete cleaning of the silica which removes all extraneous materials as organic coatings and hydration water. A new method has been developed for this process which calls for 2 weeks of oxidation with 0.1M KMnO₄, 1 week of desiccation with P₂O₅ and partial recrystallisation under vacuum at 800–1,000 °C (ref. 8). Compared with an earlier cleaning method on recent diatoms and sponge spicules^{5,8}, a +7‰ isotopic shift was observed using the improved cleaning procedure on oceanic siliceous ooze. This shift is reproducible for replicate treatments. The lower δ values obtained by the first cleaning procedure are presumably due to residual organic carbon and hydration water reacting with the silica during the high temperature heating. Results to be published elsewhere⁸ show that although the magnitude of the fractionation factor between silica and water is changed by the more complete cleaning, this does not seem to affect the variations with palaeotemperature of the isotopic fractionation. Thus, while the scale for the absolute values of the silica $\delta^{18}\text{O}$ shifts as a function of preparation, the shape of the stratigraphic isotope signal would remain unchanged. The final cleaning and the isotopic analyses were made at Centre des Faibles Radioactivités, C.N.R.S., France. Reproducibility of the isotope values was 0.1‰.

Results show an overall parallelism between the oxygen isotope records of diatoms and of the planktonic foraminifera (Fig. 1a and b). The exact time span of the record is not known, but is immaterial for the present discussion. An age of 18,000 yr for the $\delta^{18}\text{O}$ maxima⁹ near a core depth of 34 cm yields an average sedimentation rate of 1.9 cm per 1,000 yr which seems reasonable for this area. Thus, the 40 cm long core represents about 20,000 yr. The oxygen isotopes show high values in the older sediments and low values in the younger ones, reflecting the familiar transition from glacial to Holocene time. The overall range from high to low $\delta^{18}\text{O}$ values is greater for the diatoms ($\sim 1.5\%$) than for the foraminifera ($\sim 1.2\%$). The transition may be uneven^{7,10} although the exact nature of this unevenness is obscured by mixing processes.

The oxygen isotopic ratio of planktonic foraminifera in deep-sea sediments is a function of the temperature and the

isotopic composition of the water in which they developed. Part of the development can take place within the thermocline, and the average depth of the shell precipitation is thus an important factor. The isotopic composition of seawater depends on the amount of continental ice^{1,3,12} and also on the evaporation/precipitation ratio, that is, regional salinity. The interpretation of oxygen isotope values is further complicated by preferential dissolution on the sea floor of isotopically lighter shells⁷. The magnitude of these various effects has been reviewed elsewhere¹¹. Analogous effects may be expected for diatoms, except that these photosynthesising, shallow-living algae preferentially develop their skeletons within the mixed layer of the ocean. Depth of growth may, therefore, be ruled out as an important factor. Seasonal changes of temperature and changes in the evaporation/precipitation ratio will affect diatoms as much as foraminifera. Direct input of melt water could conceivably produce a differential effect on diatoms in pericontinental and high latitude waters. Any such differential is thought to be insignificant in the area under study.

The deglaciation effect accounts for most of the foraminiferal isotopic range (Fig. 1b) being approximately 1.2‰ in magnitude^{3,12}. It should influence the diatoms in a similar way. Which other effects are then responsible for the measured 0.5‰ difference (Fig. 1c) in the isotopic range between the diatoms and foraminifera?

An effect of temperature is certainly possible, if the surface water inhabited by the diatoms experienced more warming than the deeper waters inhabited by the foraminifera *Neoglobobulimina dutertrei* and *Pulleniatina obliquiloculata*. *P. obliquiloculata* secretes its shell mostly in subsurface waters whereas *N. dutertrei* typically occurs in upwelling regions and hence reflects a strong influence of cold subsurface water in its composition. Of the other possible effects two deserve special attention: a change in species composition of the diatom assemblages (season effect) and changes in the preservation of the siliceous and calcareous microfossils (dissolution effect).

With the beginning of the post glacial, upwelling seems to have been reduced in the Equatorial Pacific¹³. The abundance of *Thalassionema nitzschioides*, a diatom species especially confined to areas of upwelling, is reduced at this point. The composition of the diatom assemblages otherwise remains virtually constant throughout the core interval and any seasonal influence on the isotopic values, therefore, seems negligible.

Differential dissolution of the less silicified warm-water frustules of diatoms can enrich an assemblage with cold-water forms¹⁴ and conceivably disturb the isotopic signal. In the present case, there is no evidence for interference from such dissolution effects. Dissolution has affected certain core levels but there is no great difference between diatom preservation in glacial and post-glacial sediments in this core (Fig. 1e).

There is evidence, however, that the foraminiferal signals have been affected by differential dissolution. In the present case, such dissolution would decrease the range of the foram signal, as it removes isotopically light shells from post-glacial sediments⁷. A noticeable divergence of the signals of *N. dutertrei* and *P. obliquiloculata* begins near 17 cm in the core. A similar divergence between two foraminifera species has been shown to mark the onset of the Holocene dissolution pulse 12,500 ¹⁴C years ago⁷. A decrease in per cent carbonate immediately follows (Fig. 1d). This level also corresponds to the divergence of the diatom and foraminiferal isotope signals (Fig. 1c).

It seems, therefore, that two effects are important in producing a difference in isotopic ranges between diatoms on one hand and foraminifera on the other: (1) a greater temperature sensitivity of diatoms (because they live in surface waters) and (2) a reduction in the foraminiferal isotopic range (due to the Holocene carbonate dissolution). If differential warming of surface and subsurface water could entirely account for the discrepancy between diatoms and foraminifera, surface waters would have warmed more by 1.5 °C, about one half of the total warming calculated by CLIMAP⁹ for this area. But the difference could also be a reflection of carbonate dissolution so that the

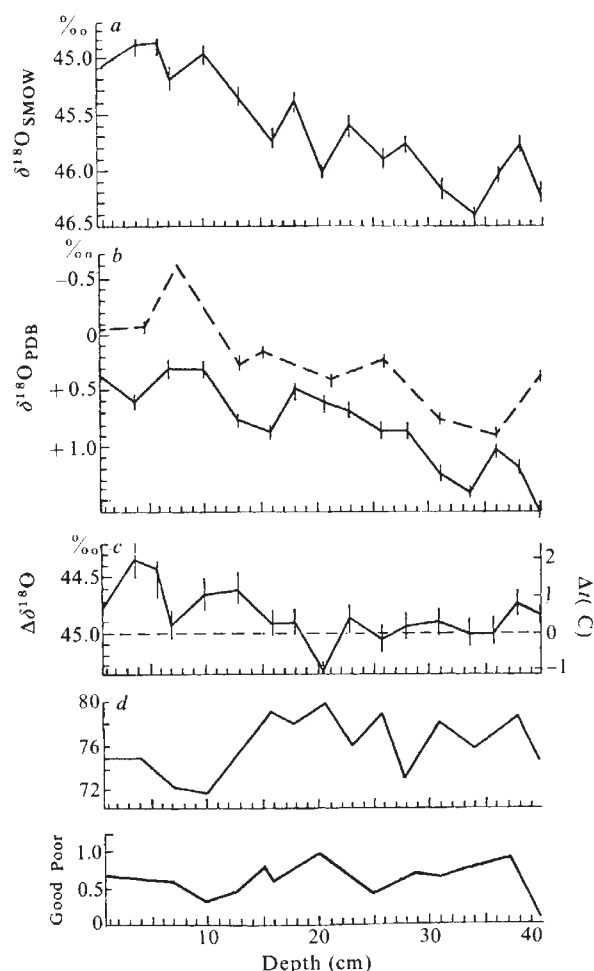


Fig. 1 Variation with depth in Core PLDS 72 Bx from the eastern Pacific of: a, $\delta^{18}\text{O}$ SiO_2 against SMOW of clean diatom valves after oxidation of organic matters with KMnO_4 , desiccation by P_2O_5 and high temperature heating (analyses by L. Labeyrie). b, $\delta^{18}\text{O}$ CaCO_3 against PDB of two foraminifera species: *Pulleniatina obliquiloculata* (dashed line) and *Neoglobobulimina dutertrei* (solid line) (analyses by J. S. Killingley and E. Vincent). c, Calculated $\Delta(\delta^{18}\text{O}) = \delta^{18}\text{O}_{\text{SiO}_2} - \delta^{18}\text{O}_{\text{CaCO}_3}$ (*N. dutertrei*). The reported Δt has been calculated from: $\Delta t \sim 4\Delta(\text{SiO}_2, \text{H}_2\text{O}) \sim 4\Delta(\text{CaCO}_3, \text{H}_2\text{O})$ (8) where zero is the mean value for the 18–40 cm depth range. d, Carbonate abundance, in per cent of weight. e, Diatom Preservation Index, a measure of the morphological change of the diatom valves caused by dissolution (analyses by N. Mikkelsen). In the relatively poorly preserved interval around 20 cm, dissolution resistant species like *Coscinodiscus nodulifer* and *Ethmodiscus rex* are concentrated¹⁶.

differential warming would be negligible.

In conclusion, the present study has shown the possibility of using the silica $^{18}\text{O}/^{16}\text{O}$ ratio in diatom valves as a palaeo-temperature indicator. The similarity between the diatom and foraminifera signals indicates that no isotopic re-equilibration between the diatom silica and the bottom water takes place. This is in contrast to findings by Mopper and Garlick¹⁵ who may have used inadequately cleaned samples. Comparison of $^{18}\text{O}/^{16}\text{O}$ ratios in diatoms and foraminifera from the same core may allow observation of changes in the thermal stratification of the oceans. The isotopic composition of diatoms will be a unique temperature tracer in high latitudes where shallow-dwelling foraminifera are commonly lacking in sediments. The isotopic composition of the diatom valves should also make it possible to extend the oxygen-isotope stratigraphy to deep-sea sediments below the compensation depth for calcium carbonate.

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Is the Hercynian Front in Ireland a local feature?

THE boundary of the northern foreland with the Hercynian orogeny in Europe—the Hercynian Front—has been recognised as a major feature in European palaeotectonic reconstructions^{1,2}, in transatlantic correlations^{3–7}, and for metallogenic models^{8–12}. In all these assessments the Hercynian Front (or Thrust Front) is depicted as a structural line transecting southern Ireland on a crudely east-south-east (American) trend across to South Wales. It is suggested here, however, that the 'Front' in Ireland has no direct link with South Wales, being separated by a major basement block, and may well be a localised internal foreland feature.

The position of the Hercynian Front in Ireland is based on the perceptive study by Gill¹³, who recognised a marked change in tectonic style across an arcuate line from Killarney to Dungarvan, part of which consisted of northerly directed thrusts. This he termed the Thrust Front, which was subsequently^{14,15} modified to continue westwards through Dingle Bay (Fig. 1). The acceptance of the line of the 'Front' here has led to recent attempts to trace it off the west coast of Ireland¹⁶ and to suggest a link with the Gibbs Fracture Zone^{5,6}.

An examination of the Hercynian fold trends in southern Ireland¹⁴, however, reveals three aspects at variance with the popular conception. First, south of the 'Front' the folds show an arcuate trace markedly divergent from the east-south-east American trend except in the Dungarvan area; second, from Dungarvan eastwards the trend rotates back to an east-north-east direction (Fig. 2), negating the simple east-south-east extrapolation directly to South Wales; and third, the strike of Hercynian structures in the south of Ireland show close agreement with Lower Palaeozoic structural trends except to the north of Dungarvan, and hence can be regarded as having a Caledonoid trend rather than an Armorican one.

On this basis the concept of an American trending Hercynian imprint on the south of Ireland, which transects

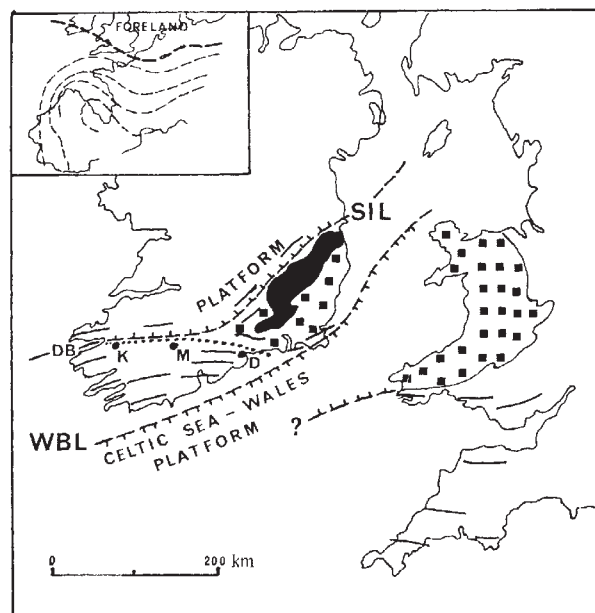


Fig. 1 Sketch map of Hercynian fold trends in Southern Ireland and south-west Great Britain. Blocked areas represent Lower Palaeozoic massifs, with the Leinster pluton and its concealed extension shown in solid. Dotted line indicates position of Hercynian Front in Ireland^{13,15}. SIL, South Ireland Lineament; WBL, Wexford Boundary Lineament; DB Dingle Bay; K, Killarney; M, Mallow; D, Dungarvan. Data sources from refs 14,25,26. Inset map showing location of Hercynian orogenic zones in Western Europe^{11,12} on a pre-Permian reconstruction²⁷.

earlier Caledonoid trends and can be matched directly with that of south-west Wales and south-west England, cannot be upheld. Rather, the Hercynian structural pattern here is considered to have been locally basement controlled, and to reflect the influence of three major basement elements which resulted in contrasting 'platform' and 'basin' (graben) deformation¹⁷. These major elements are the South Ireland Lineament, the Wexford Boundary Lineament, and the Leinster pluton (Fig. 1). These two lineaments, which parallel Caledonoid structures, have been recently recognised as major temporally persistent palaeo features, influencing lower Palaeozoic deposition and deformation¹⁸ as block boundaries. Between, and controlled by, these two lineaments there was subsequently a thick Upper Palaeozoic accumulation (>4,000 m) in a structural basin¹⁹, thinning dramatically eastwards onto the positive block of the Caledonoid Leinster unit stiffened by its mid-Devonian pluton (Fig. 2).

Because of this eastward shallowing basement in the sector between the two lineaments, Hercynian compression here resulted in two differing structural provinces, a south-west 'graben' province, and a north-east 'platform' province. The former shows polyphase fold deformation^{13,20} with tight major fold structures and a locally penetrative cleavage fabric, together with severe northerly directed thrusting and structural dislocation in the Killarney–Dingle Bay area^{15,20} along the margin of the block delineated by the South Ireland lineament, and also further south at Kenmare²⁰ and Bantry (Gardiner, unpublished). Folds parallel the Caledonoid trend of this lineament, predictably swinging to an east-south-east orientation as deformation was influenced by the positive Leinster block¹³ in the Mallow–Dungarvan area. In contrast, in the Leinster platform area the Upper Palaeozoic cover shows only broad open folds due to the basement protection, and the folds are moulded against the Leinster pluton (Fig. 2). Post-Caledonian flexing of the Lower Palaeozoics in south-west County Wexford²¹ and the inferred present day strike

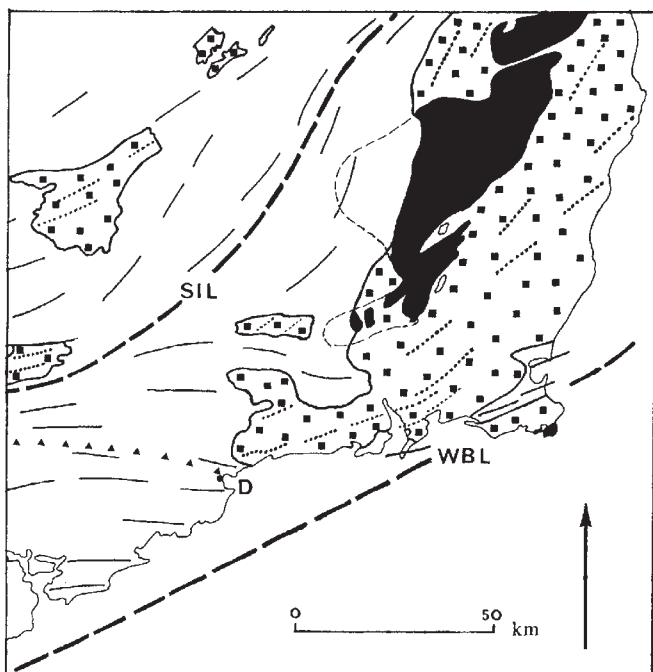
swings of the two lineaments are also thought to be due to late stage Hercynian tightening against the rigid Leinster pluton.

In this structural model the northern boundary of the zone of 'graben' deformation therefore has an arcuate trace (Fig. 1) which is essentially the line of the recognised 'Thrust Front'¹¹⁻¹⁵. The major tectonic dislocations recorded at Mallow and further west^{13,15} seem, however, to be relatively localised features reflecting proximity to the edge of the northern positive block. Their apparently problematical absence east of Mallow is entirely predictable on this model, as this was a transitional zone between the 'graben' and 'platform' deformation areas, with deformation intensity decreasing northeastwards due to the progressive basement influence of the lower Palaeozoic Leinster block. This factor was recognised by Philcox²², who suggested that east of Mallow the 'Thrust Front' was split into a number of fanned 'arms' as it was accommodated by the Upper Palaeozoic cover above interfering basement units. Certainly there is no one continuous 'Thrust Front' in this area²³, as was earlier proposed¹³, and use of this term here would seem unsuitable.

Recognition of a positive basement block between south-east Ireland and South Wales during the Hercynian orogeny also creates problems in any simple linkage of the Hercynian Front between these two areas. Indeed, the Welsh Lower Palaeozoic massif seems to have also been part of this intervening structural platform, as the Hercynian fold trends in South Wales seem to have been influenced by the arcuate southern margin of the massif²⁵ (Fig. 1). The 'Thrust Front' position in south-west Wales is also a reflection of basement-cover interaction^{4,24}, although its position further southeastwards is by no means clear²³.

It is, therefore, concluded that there is no continuous linkage between the Hercynian Front in south-west Wales and Southern Ireland, in both areas its location and trend

Fig. 2 Sketch map of south-east Ireland, showing Caledonoid and Hercynian fold trends and the inferred positions of the South Ireland and Wexford Boundary Lineaments. Granites are in solid, with concealed extension of Leinster pluton shown by dashed line. Lower Palaeozoic and pre-Cambrian areas are blocked. ▲ show line of the Hercynian Front as recognised by Gill¹³. Other data sources from refs 14, 21, 26, 28.



being a function of basement control; simple metallogenic correlations are, therefore, improbable. Transatlantic correlation seems likely to relate to pre-Hercynian structural elements, and will depend on whether the western continuation of the 'Front' with the northern foreland is in fact along the South Ireland lineament or the southern margin of the Celtic Sea-Wales platform block (Fig. 1). If the latter, then the 'Thrust Front' in Ireland is perhaps a localised internal foreland feature. Certainly recent work off the west coast of Ireland¹⁶ indicates that the Gibbs Fracture zone is better related to the Great Glen Fault line rather than to the 'Hercynian Front' in Ireland. The inferred westerly closing of the other Hercynian orogenic zones in Western Europe when plotted on a pre-Permian reconstruction^{1,11,12} (Fig. 1, inset) would also accord with a continuation of the true Front southwestwards from south-west Wales. There is no evidence of proximity to a plate boundary directly off the south of Ireland, and the inferred evolutionary history of this area^{18,19} indicates that in Hercynian times there was no oceanic crust north of the Celtic Sea-Wales platform.

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Song repertoires and territory defence in the great tit

BIRD song is generally considered to have one or both of two main functions: attracting a mate and proclaiming a territory. The great tit (*Parus major*) is a typical songbird in that song is primarily a male vocalisation and it is produced before and during the breeding season (that is, from January to May in southern England). We have shown that great tit song plays a part in territory maintenance by experiments in which we 'occupied' territories with loudspeakers after removing resident pairs^{1,2}. New pairs seeking an empty space in which to settle moved into control areas more rapidly than into song-occupied territories. We report here on three experiments designed to study the effect of a varied song repertoire in territory maintenance. Each male great tit, in common with many other songbird species, has a repertoire of song variants some of which are usually shared between birds in a locality. In the great tit, repertoire size commonly varies between two and eight song types. In some species, for example the canary, elaborate repertoires seem to be favoured by sexual selec-

Table 1 Results from Marley Wood near Oxford showing that there is no evidence for female choice of males with large repertoires

Year	<i>N</i>	Repertoire size	
		1-3	4-6
1975	Laying date*	31.9 $\pm$ 0.62	35.6 $\pm$ 3.5
	Clutch size*	7.7 $\pm$ 0.21	7.3 $\pm$ 0.5
	Per cent adult	59	50
1976	<i>N</i>	8	5
	Laying date	32.7 $\pm$ 2.03	28.6 $\pm$ 2.82
	Clutch size	8.1 $\pm$ 0.44	8.2 $\pm$ 0.58
	Per cent adult	75	60

This result is not surprising since females choose mates before the spring peak of male song. If females did prefer males with larger repertoire sizes, one would predict a correlation between repertoire size and breeding date or female fecundity. The age-corrected data show no such trend. The table also shows that males with larger repertoires do not tend to be older.

*Corrected for age of ♀

†Time in days after April 1.

tion^{3,4}, but we have found no evidence for this in the great tit (Table 1), in which pairing takes place before the spring peak of song. We therefore tested the possibility that repertoires enhance the effectiveness of song as a territorial display.

The three experiments were all of similar design. We plotted the territories of all the pairs (which were colour ringed) in a 6 hectare piece of mixed deciduous woodland² (Higgins Copse) near Oxford in early spring. In each experiment the wood was divided into three areas: control, single song and repertoire. At the start of an experiment we captured (under licence) all the resident birds in the wood during the first two or three hours after dawn on one day. The control area was left empty, the single song and repertoire areas were occupied by loudspeakers broadcasting either a single song type or a varied repertoire of songs. The broadcasting system consisted of a Uher 4000 IC tape recorder equipped with a 'Cousino' endless loop tape linked by a 15-W amplifier to four Goodman's Midax

metal horns. Each metal horn was located 50 m from the tape recorder in a different part of the 'occupied' territory. The system was powered by a 12 V battery and it operated on a time schedule controlled by a Crouzet 24 h time clock linked to a Crouzet 30 min cam timer.

The timer was set so that song was played for two minutes from each of the four loudspeakers in turn (a total of eight minutes of song). The eight minute cycle was triggered by the 24 h time clock at intervals throughout the day from dawn till dusk. During the first 3 h after dawn, the cycle was triggered once every 30 min, and for the rest of the day once an hour (simulating the natural morning peak of song). In experiments *a* and *c* (Table 2) there was one broadcasting set-up in each experimental territory; in experiment *b*, the wood was simply divided into three areas without regard to the previous territory boundaries, and the two experimental areas were occupied by a single tape recorder linked to six loudspeakers. To control for variations in attractiveness of different parts of the wood, we altered the positions of the treatment areas between experiments.

The broadcast songs were recorded from the local birds which had previously occupied territories in the wood, or from birds occupying territories in a nearby wood. We had previously made a comparison of the effectiveness in deterring invaders, of songs from within the wood and songs from nearby woods, and found that there was no difference: in four paired comparisons occupied by 'native' and 'nearby' repertoires, the following results emerged; on one occasion, the territory occupied by native songs was invaded first, on another, the other territory was invaded more quickly, and on two occasions there was no difference in latency to occupation. The repertoires used in the present experiments consisted either of local birds' song or a mixture of local and other birds' songs, and in the single song treatments we used local birds' songs.

Throughout each experiment, the pattern of reoccupation of the wood by newcomers was monitored by continuous observation from dawn till dusk. The locations of all new birds were marked on a map with reference to grid posts, and all parts of the wood were visited at least once each hour. The experiment was deemed to have ended when the

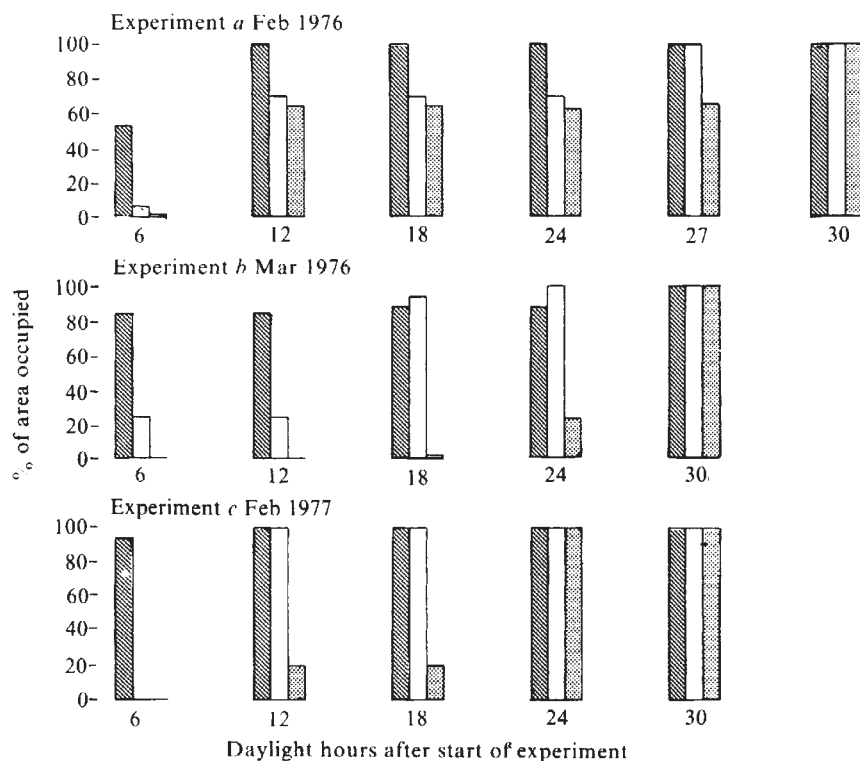


Fig. 1 A summary of the results of the three experiments. The graphs show the cumulative proportion of the three areas occupied since the start of the experiment after different numbers of daylight hours. Cross-hatched columns, control; open columns, single song; stippled columns, repertoire.

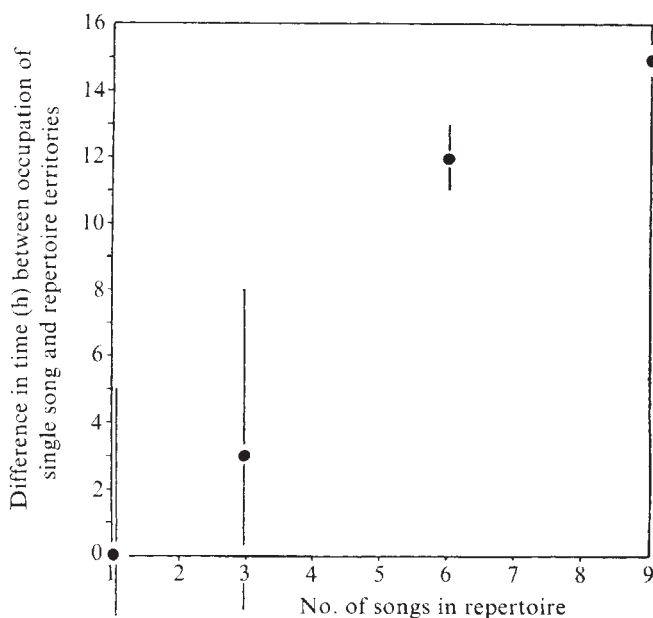


Fig. 2 The difference (in daylight hours) in the time to 90% occupation of repertoire and single song areas in relation to repertoire size. The vertical bars are standard errors. The reason for plotting the difference rather than absolute time for occupancy is that the total time span of the experiment differed between the three replicates. We took 90% of the area occupied as a criterion of 'occupation' because it was impossible to distinguish in our observations between 90% and 100% occupancy.

wood was completely occupied by new birds, at which point the speakers were turned off.

The results are summarised in Figs 1 and 2, and Fig. 3 shows an example of the reoccupation pattern over successive days. Figure 1, in which we plot the cumulative proportion of the wood occupied by new birds as a function of time, shows that in all three experiments the sequence of reoccupation of areas was: control, single song and repertoire. The three experiments were performed with repertoires of different sizes spanning the normally occurring range: 3, 9 and 6 in experiments *a*, *b* and *c*, respectively. Figure 2 shows that the difference between the time to 90% occupation of the repertoire and single song areas increases with increasing size of repertoire. We suggest that song repertoires enhance the effectiveness of song as a 'keep out' signal, and that larger repertoires are more effective than small ones. In view of this latter finding, it is surprising that the commonest repertoire sizes in our study area are 3 and 4 song types.

One or both of two mechanisms might account for our results. First, the various songs in the repertoire could be used in slightly different contexts. Three possible contexts are: time of the year, position in the territory, and the particular rival at whom the song is directed. We tested

whether or not birds use different song types at different stages in the season of territory establishment by comparing the relative frequency of use of songs during two sample periods separated by about two weeks (late January versus mid-February for three birds, and late February versus mid-March for three others). The correlation coefficient based on all six birds was highly significant ($P < 0.001$), suggesting that relative frequency of use of songs in the repertoire does not vary with season. We also did a similar correlation analysis of the relative use of different song types in different parts of an individual's territory by dividing each of the same six territories arbitrarily into north and south halves: again the correlation was highly significant ($P < 0.001$). This analysis was performed because the different songs in a repertoire might be adapted for use in the specific acoustic climate of different parts of the territory, and it is still possible that a more detailed analysis would support this idea, although our present results do not. One contextual factor which does influence the use of song types in a repertoire is the song of intruding rivals: resident birds tend to match the songs of intruders (J.K. & R.A., in preparation). Matching seems to act as a way of indicating that the song is directed at a particular intruder (who then retreats), and clearly a bird with a repertoire of songs is better able to match. This was not a major contribution to our results, however, as the loud-speakers in our experiments were not specifically programmed to match the songs of invaders.

The second mechanism which might account for our results is the so-called Beau Geste effect¹. It is based on the fact that, as our experiments show, birds attempting to establish a territory use song as a cue in choosing where to settle. Reproductive success in woodland in the great tit

Fig. 3 An example of the results of one experiment (experiment *b*). The four maps show the sequence of reoccupation of the wood and the division of the wood into three treatment areas. Key: broken lines, division into treatments; large dots, new territorial pair in control area; small dots, new pair in repertoire area; cross-hatching, new pair in single song area; *a-f* represents the sequence of arrival of the new birds (*b* disappeared after day 2). Scale 6 mm, 50 m.

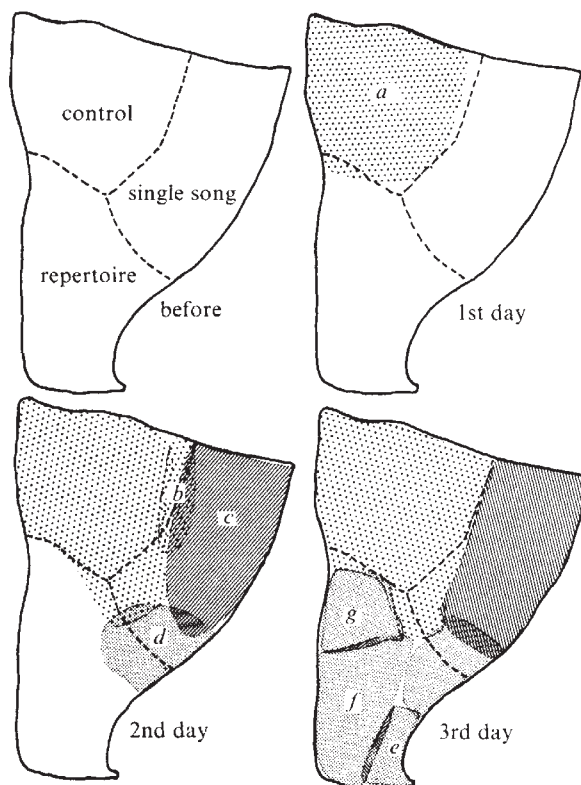


Table 2 A summary of the three experiments reported in this paper

Experiment	Date	No. of territories before			No. of pairs reoccupying the wood
		R	S	C*	
<i>a</i>	Feb 23-25 1976	3	2	3	4
<i>b</i>	Mar 15-18 1976	4†			4
<i>c</i>	Feb 15-18 1977	2	2	2	6

*Repertoire, single song, control.

†In this experiment the 3 treatments were assigned without regard to previous boundaries (see text).

Table 3 Data showing that a male is much more likely to change song perches at the same time as changing song types

Probability of changing perches <i>N</i>	Next song burst	
	Same song type	Different song type
	0.035	0.44
	1,755	18
	$\chi^2 = 79.95$	

The data were collected in an open parkland area (University Parks, Oxford), and changing song perches was defined as changing trees (the birds often hop about continually within a tree). The data only refer to changes in perch or song type within a period of song (that is, the bird did not pause for longer than 30 s between song bursts).

is negatively correlated with density⁶, so a settler should avoid crowded woodlands. Residents might therefore discourage invaders by singing several different songs, and creating the impression of a crowded habitat. The fact that territorial birds tend to change perches when they change song type (Table 3) is consistent with this ideal. Of course the deception we are proposing would only work to the extent that potential settlers do not correct for repertoire size.

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Tolerance of *Triticale*, wheat and rye to copper deficiency

THE aim in producing *Triticale* was to combine the hardiness and tolerance of low soil fertility of rye with the superior yield and bread-making qualities of wheat¹, but there has been little experimental evidence that triticales carry the desirable characteristics from rye². We present here evidence of the tolerance of triticale to low concentrations of available copper in soil, a condition widely associated with poor sandy soils in Australia³. Such soils may contain enough total copper for tens of thousands of crops but it is relatively unavailable to widely grown cultivars of wheat, oats and barley. In contrast, rye rarely shows a response to copper on these soils^{4–7}.

Triticales are hybrids of wheat and rye and there are two major classes: hexaploids, the most promising agriculturally, are derived from crosses between diploid rye (*Secale cereale*) and tetraploid wheat (*Triticum durum*), and octoploids come from crosses between rye and hexaploid wheat (*Triticum aestivum*). In our study a hexaploid triticale and an octoploid triticale and their parent genotypes were grown in pots of copper-deficient soil with four levels of copper supplied. Chinese Spring wheat and Imperial rye are known to be the parents of the octoploid triticale⁸ but the parents of the hexaploid triticale Beagle are not known, it having resulted from crosses between many parent genotypes⁹. Accordingly, the genotypes chosen to represent the parents of the hexaploid in our study are approximate only, though

certainly, together with Chinese Spring, their response to the level of copper supplied is characteristic of their species^{4–7}.

Grain yields for each of the five genotypes at each level of copper are shown in Table 1. Both wheats were extremely sensitive to the level supplied, producing no grain without supplementary copper. In contrast, rye and the hexaploid triticale were remarkably tolerant of this soil, producing as much grain in untreated soil as when copper was added. The mean grain yield of the Imperial rye was unusually low because of its poor adaptation to the environment; it matured four weeks later than the other genotypes under high temperatures and consequently grain numbers were low and grain size was small although, like grain yield, neither grain number nor size responded to copper application.

Clearly hexaploid triticale has followed its rye parentage in its tolerance of low copper supply; its yield without added copper was comparable to that of wheat in soil with the highest level of added copper. The most immediate explanation for the gross differences in response to copper supply seems to be male sterility. Copper deficiency causes male sterility in wheat¹⁰ and both pollen viability and grain number (D. T. Pearce and R.D.G., unpublished results) have shown the same pattern of response to copper supply as did grain yield (Table 1).

The difference in response of wheat and rye in copper-deficient soils is due to the ability of rye to maintain a higher concentration of copper in the shoot^{4–7,11}. Concentrations of copper in triticale seem to be intermediate between wheat and rye (unpublished results) but sufficient to maintain the plant above the threshold which prevents male sterility. Rye has a more extensive root system than wheat¹² but the higher levels of copper may be due to a more specific character than size of root system because in a study by Piper and Walkley¹¹, rye contained more copper but not more zinc.

Rye and triticale may thus be termed copper-efficient and wheat copper-inefficient. The difference in the responsiveness of octoploid and hexaploid triticale may reflect the degree of buffering of copper efficiency of the rye genome by different numbers of wheat genomes. Two genomes of tetraploid wheat retain high efficiency whereas three

Table 1 Effect of copper supply and genotype on yield of grain in a copper-deficient sandy soil

Genotype	Ploidy	Copper supply (mg per pot)			
		0	0.1	0.4	4.0
		Grain yield* (g per pot)			
<i>Triticum durum</i> cv Cocorit	Tetraploid	0	0.1	5.6	12.5
<i>Triticum aestivum</i> cv Chinese Spring	Hexaploid	0	3.5	12.9	13.7
<i>Secale cereale</i> cv Imperial	Diploid	2.1	2.4	2.4	2.1
<i>Triticale</i> cv Beagle	Hexaploid	14.3	13.8	14.3	14.5
<i>Triticale</i> Chinese Sp.-Imperial	Octoploid	4.6	6.5	7.4	7.5

*Each value is the mean of three replicates. Plants were grown in a copper-deficient siliceous sand from Tintinara, South Australia (Laffer Sand) in an evaporatively cooled glasshouse. Each pot contained 12 kg of soil which supported three plants. Appropriate amounts of nutrients N, P, K, Ca, Mg, S, Fe, B, Mn, Zn, Mo, Co and Cl were added with or without Cu. Of the genotypes, only wheat plants failed to grow normally in the soil without added Cu, showing characteristic symptoms of wilting, delayed maturity and failure to set grain¹³. The copper responses of both wheats and the octoploid triticale are significant at $P = 0.001$.

genomes of hexaploid wheat reduce the efficiency to a position intermediate to hexaploid triticale and hexaploid wheat.

Several nutritional characters have been shown to be under single-gene control, for example, iron efficiency in soybeans¹³ and boron efficiency in tomato¹⁴, and this may also be the case for copper. Pugsley (personal communication) in a hybridisation study of copper-efficient oats (*Avena strigosa*) and copper-inefficient oats (*A. sativa*) has found copper efficiency to be controlled by one or two genes. Triticale cultivar Beagle used in my study contains the complete rye genome of seven chromosome pairs⁹, but studies in progress suggest that copper efficiency is carried on a single rye chromosome, and if this is so, it should be possible to translocate the relevant part of the chromosome to wheat to produce a copper-efficient line of wheat.

Copper efficiency from rye is transferable to triticale and the potential of this new crop for marginal lands deserves further examination. The mechanism of enhanced copper utilisation is important too, for if it is linked with the morphology of the root system, it may influence the utilisation of other immobile soil nutrients.

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Activation of an endogenous retrovirus from *Tupaia* (tree shrew)

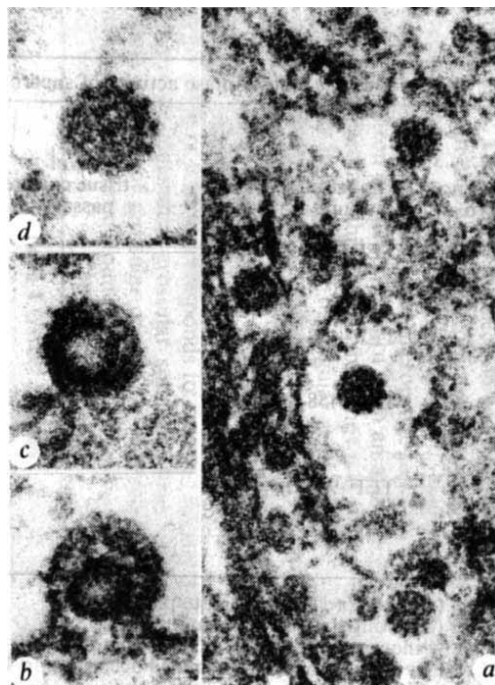
TYPE-C RNA viruses have been isolated from tissues and cell lines of various mammalian species, including mice^{1,2}, domestic cats³, pigs^{4,5}, woolly monkeys^{6,7}, gibbons⁸ and squirrel monkeys⁹. The viruses from woolly monkeys seem to be horizontally transmitted as infectious agents which cause naturally occurring tumours in gibbons⁷. Apart from the known aetiological role of certain oncornaviruses in the formation of neoplasms, there has been increasing interest in the isolation of mammalian retroviruses and their evolutionary relatedness to different species. Experimental evidence suggests that during evolution an endogenous virus crossed from one species to another¹⁰. *Tupaia* (the tree shrew), a member of the family Tupaiidae, is regarded as one of the most primitive living prosimians, bridging the gap between insectivores and primates^{11,12}. We report here the detection and activation, *in vitro* and *in vivo*, of an endogenous retrovirus from *Tupaia*. This opens up the possibility of a direct comparison of the *Tupaia* retrovirus with type-C viruses from rodents and primates.

Reports^{13–15} on type-C RNA viruses in placentae of normal, healthy Sprague-Dawley rats initiated a search for an analogous *Tupaia* virus in placentae of healthy, untreated animals and others treated with iododeoxyuridine (IUDR) 24 h before the end of pregnancy. We used *Tupaia belangeri* Baf: BIAS (Baf designates the breeder, the Battelle-Institut, Frankfurt and BIAS is the name of the stock used¹⁶). Placentae were

removed aseptically from untreated, healthy, pregnant *Tupaia* at full term and prepared for electron microscopy. Figure 1 shows an electron photomicrograph of virions, which in size and structure are morphologically similar to type-C virus particles. Figure 1b shows a budding virus particle; some virions have spike-like structures consistent with reports that the ultrastructure of type-C virus particles varies considerably^{17,18}.

We took a more direct approach by establishing *Tupaia* fibroblasts from skin of single embryos or juvenile animals in tissue culture. Some cultures were treated with IUDR to activate retrovirus. We describe here the experiments with the cell cultures derived from the second embryo of one animal (no. 458, TEF-458-2). The cells were established and propagated in tissue culture as before¹⁹. They were grown at 37 °C and treated with IUDR in the replication log phase of cell growth. After treatment with IUDR (50–200 µg ml⁻¹) for 24 h several clones were isolated and established in tissue culture. Treatment of *Tupaia* fibroblasts with 200 µg IUDR per ml of medium had no highly toxic effect within 24 h. The plating efficiency of the treated cells (measured according to Ham²⁰) decreased to 17% that of untreated cells and was 22.5%. The supernatants of the different cell clones were assayed for reverse transcriptase activity^{21,22}. Poly (rA)·(dT)₁₄ served as template/primer; poly (dA)·(dT)₁₄ was used as a control²³. The incorporation of ³H-dTMP was maximal in the presence of 0.5 mM Mn²⁺ as divalent cation (Fig. 2). In the presence of poly (dA)·oligo (dT) only small or negligible levels of RNA-directed DNA polymerase (RDDP) activity were observed. RDDP activity detectable with poly (rA)·oligo (dT) was inhibited by 2 mM inorganic phosphate, which is characteristic for mammalian virus DNA polymerase²⁴. Low levels of ³H-dTMP incorporation were observed in supernatants of *Tupaia* cell cultures not treated with IUDR, whereas an increase

Fig. 1 Electron micrographs of ultrathin sections of *Tupaia belangeri* placenta. The placenta tissue was fixed in 2% glutaraldehyde, buffered with 0.05 M sodium cacodylate, pH 7.2, in the cold for 20 min, followed by several washes with cold buffer and osmication in the cold (2% osmium tetroxide). The material was dehydrated through graded ethanol solutions and embedded in Epon 812. *a*, Virus particles in *Tupaia* placenta (the bar represents 200 nm). *b*, Virus particle budding like type-C virus from *Tupaia* placenta (the bar represents 100 nm); the centrally located nucleocapsid is in direct contact with viral envelope (the bar represents 100 nm). *c*, The cylindrical particle has an internal structure similar to that of the immature type-C particle. *d*, Extracellular, immature virus particle (the bar represents 100 nm).



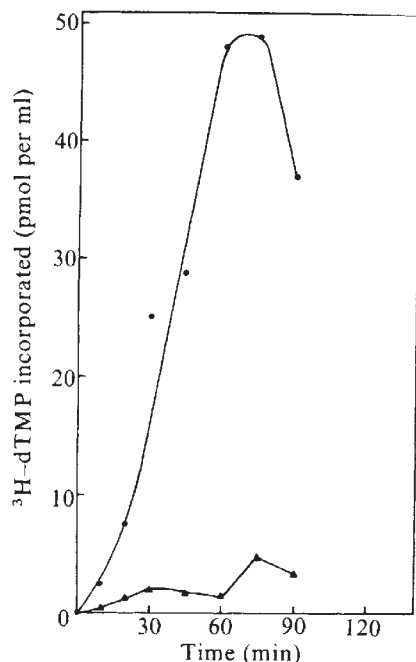


Fig. 2 Kinetics of polymer synthesis with reverse transcriptase from supernatants of TEF-458-2-J-2 cell cultures using poly (rA) · oligo (dT) (●) or poly (dA) · oligo (dT) (▲) as template/primer. The reaction mixtures (0.10 ml) consisted of 50 mM Tris-HCl, pH 8.1, 30 mM KCl, 0.5 mM MnCl₂, 1 mM dithiothreitol, 0.08 mM dTTP including ³H-dTTP (Radiochemicals Centre, Amersham, specific activity 62 Ci mmol⁻¹), poly (rA) · (dT)₁₄ at 48 μM (Collaborative Research, Waltham, Massachusetts). Reactions were started by the addition of 60 μl viral suspension (disrupted by Nonidet P-40 for 10 min at 24 °C) to the polymerisation mixtures. Reactions were carried out at 37 °C and monitored as described before²³.

in RDDP activity was observed routinely in cultures treated with halogenated pyrimidines (Table 1). All cell cultures used for the DNA polymerase assays (Table 1) were of either the same or nearly the same passage level.

To activate retrovirus *in vivo* from *Tupaia*, a pregnant animal (no. 944, 205 g body weight) was injected intraperitoneally with IUdR (10 μg per g body weight) on the 35th day of gestation—the normal period of gestation being 44 ± 1 d. Embryo no. 3 was removed aseptically by caesarean section 24 h later, and skin fibroblasts were explanted and

established in culture. RDDP activity (Table 1) revealed an increase in ³H-dTMP incorporation for TEF-944-3-J compared with the low levels found in supernatants of TEF-944-1 cultures derived from embryo no. 1 which had been removed before the injection of the mother animal no. 944 with IUdR.

To detect viral RNA we used ³H-uridine (specific activity 58 Ci mmol⁻¹). After phenol extraction²⁵ a peak of radioactivity banded at 70S after 5–20% sucrose gradient centrifugation (RNA from Gross strain of MuLV served as marker). When subjected to heat denaturation (3 min at 100 °C with 2% sodium dodecyl sulphate) the 70S RNA was converted into a broad peak centred around 35S. Furthermore, ³H-uridine-labelled virions from supernatants of three *Tupaia* cell cultures (TEF-944-3-J, TEF-458-2-J-2 and TEF 458-2-J-2-J) were isolated after purification by low and high speed centrifugation and banded in 15–55% neutral sucrose gradient. The activity peak banded at a density of 1.15–1.17 g cm⁻³—characteristic for retroviruses²⁶ (Fig. 3).

In a host range study, the isolated *Tupaia* retrovirus (TRV-1) was inoculated into human embryonic lung fibroblasts (HELFI)²⁷, human foreskin fibroblasts (HFF-3)²⁸, marmoset skin fibroblasts (HF)²⁹, mink lung cells (Mv1Lu)³⁰ and dog thymus cells (Fcf2th)³⁰. RDDP activity was detected in culture fluid from inoculated canine cells only at a low level. Cocultivation of isolated *Tupaia* cell clones (TEF-944-3-J, TEF-458-2-J-2 and TEF-458-2-J-2-J) with XC cells³¹ did not result in syncytia formation. Cocultivation of XC cells with Fcf2th cells infected with TRV-1 resulted in the formation of multinucleated giant cells containing 10–20 nuclei. The lack of formation of giant cells after cultivation of producer cell clones (TEF-458-2-J-2, TEF-944-3-J and TEF-458-2-J-2-J) with XC cells could indicate that TRV-1 is not of murine origin. However, the lack of induction of XC syncytia does not rule out a murine origin for TRV-1, because some murine leukaemia viruses do not induce XC syncytia³². Cytotoxicity tests with *Tupaia* producer cell clones and anti SSV-1 sera gave negative results. This suggests that SSV-1-specific surface receptors are not present in the tree shrew cell clones.

The absence of RDDP activity in normal *Tupaia* cells, and the inducibility by IUdR of a retrovirus from three different tree shrew cell cultures, suggest that TRV-1 is an endogenous retrovirus. Our data represent the first isolation (as far as we know) of an oncornavirus in the primitive prosimian *Tupaia*.

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Table 1 Reverse transcriptase activity of supernatants of normal and IUdR-treated *Tupaia* skin fibroblast cell cultures

Code no. of animal/no. of embryo	Label of culture	No. of tissue culture passages	Treatment with IUdR	³ H-dTMP incorporated (pmol per μg protein)*	³ H-dTMP incorporated (pmol per μg protein)†	(rA) — (dA)
458/2	TEF-458-2	2	—	0.12	0.19	0.63
		3–7	—	0.10–0.46	0.15–0.65	0.67–0.71
		17	—	0.18	0.24	0.75
		25	—	0.43	0.61	0.70
	TEF-458-2-J-2	8	+	4.22	0.19	22.2
		11	+	6.19	0.16	38.7
		13	+	5.14	0.22	23.4
	TEF-458-2-J-2-J	8	+	8.57	0.21	40.8
		11	+	7.86	0.17	46.2
		13	+	5.74	0.25	22.9
944/1	TEF-944-1	2	—	0.16	0.20	0.8
		3–5	—	0.19–0.66	0.20–0.62	0.95–1.0
944/3	TEF-944-3-J	2	+	2.11	0.26	8.2
		5	+	5.75	0.20	28.8

*With poly (rA) · oligo(dT) as template/primer.

†With poly (dA) · oligo(dT) as template/primer.

Figures show incorporation of ³H-dTMP (pmol per 30 min) at 37 °C into acid-insoluble product. Assays were performed as described in the legend to Fig. 2. Protein was determined according to Lowry's³³ method.

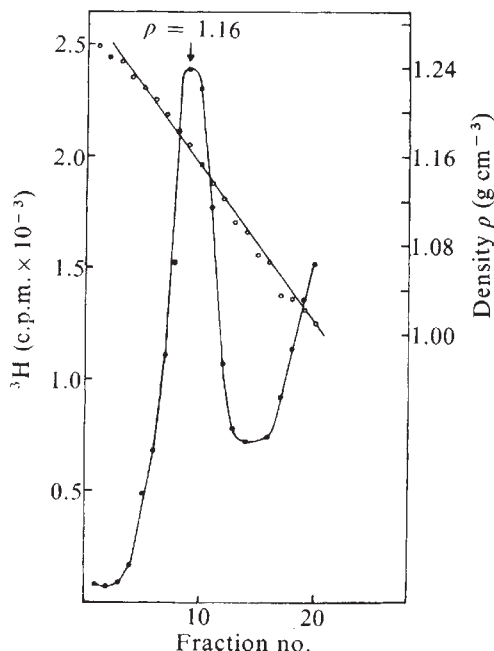


Fig. 3 Equilibrium gradient centrifugation of TRV-1 virions. Culture fluids from TEF-458-2-J-2 cells incubated with ^3H -uridine ($28 \mu\text{Ci ml}^{-1}$) were clarified by centrifuging at $1,000g$ for 20 min and centrifuged through a 5% (w/v) cushion of sucrose for 60 min at $25,000 \text{ r.p.m.}$ in a SW27 rotor. The pellet was resuspended in 0.1 M NaCl , 10 mM Tris-HCl , $\text{pH } 7.2$, and 1 mM EDTA and layered on a 5-ml linear 30–60% sucrose gradient in the same buffer. It was then centrifuged at $50,000 \text{ r.p.m.}$ in a SW65 rotor for 4 h at 4°C . The gradient was fractionated and each fraction was assayed for acid-precipitable radioactivity (●) and density (○).

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5'-AMP is a direct precursor of cytokinin in *Dictyostelium discoideum*

ISOPENTENYLADENINE ($i^6\text{Ade}$) and closely related derivatives occur naturally either as free bases or as constituents of tRNA^{1,2}. Although these cytokinins have been detected in various organisms, it is still not clear whether they are produced by the degradation of tRNA or whether they are synthesised by some pathway not involving tRNA. A spore germination inhibitor, discadenine, isolated from *Dictyostelium discoideum* was found to be an $i^6\text{Ade}$ derivative with a 3-amino-3-carboxypropyl group in the 3 position³—the cells contained a large quantity of free $i^6\text{Ade}$, presumably a precursor of discadenine⁴.

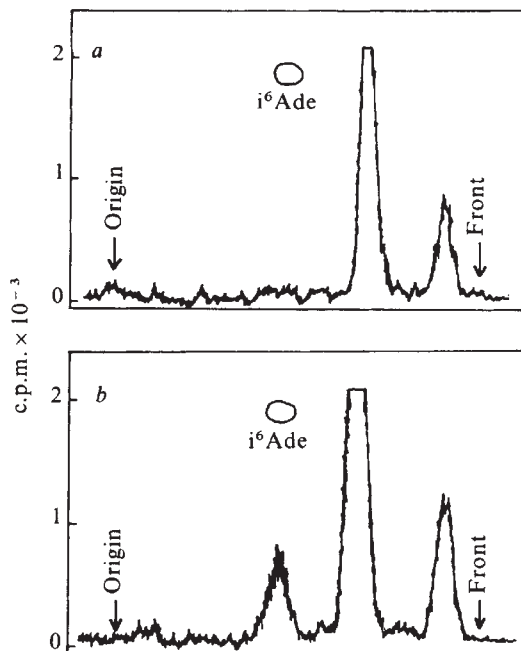


Fig. 1 Thin-layer chromatography of the products synthesised *in vitro* using ^{14}C -isopentenylpyrophosphate in the absence (a) or presence (b) of the supernatant of nondialysed, boiled S-100. The reaction mixture contained 20 mM Tris-HCl ($\text{pH } 7.5$), 5 mM magnesium acetate, $0.05 \mu\text{Ci}$ of ^{14}C - Δ^3 -isopentenylpyrophosphate (57 mCi mmol^{-1} ; Radiochemical Centre), 0.5 mg of dialysed S-100 extract and $10 \mu\text{l}$ of the supernatant of nondialysed, boiled S-100 in a final volume of $100 \mu\text{l}$. After 60 min incubation at 27°C , $150 \mu\text{l}$ of ethylacetate was added and the mixture was then vigorously shaken and centrifuged. The resultant upper layer was spotted on a plate of silica gel (Merck) and developed (10 cm) with chloroform-acetic acid (9:1, v/v). Chromatograms were scanned with a radiochromatogram scanner (Packard 385). Slit width was 2.5 mm . The S-100 extract was prepared from late culmination cells of *D. discoideum* strain NC-4 as follows; 1 g of cells were ground with 2 g of alumina, and extracted with 5 ml of buffer A (10 mM Tris-HCl , $\text{pH } 8.0$, 10 mM magnesium acetate, 60 mM KCl , 1 mM EDTA , 6 mM 2-mercaptoethanol and 10% glycerol). The extract was centrifuged at $13,000g$ for 30 min and the resulting supernatant was then further centrifuged at $156,000g$ for 60 min. The supernatant obtained was dialysed overnight against two changes of buffer A (300 ml each). The supernatant of nondialysed, boiled S-100 was prepared by boiling the nondialysed S-100 ($25 \text{ mg protein ml}^{-1}$) for 2 min and centrifugation.

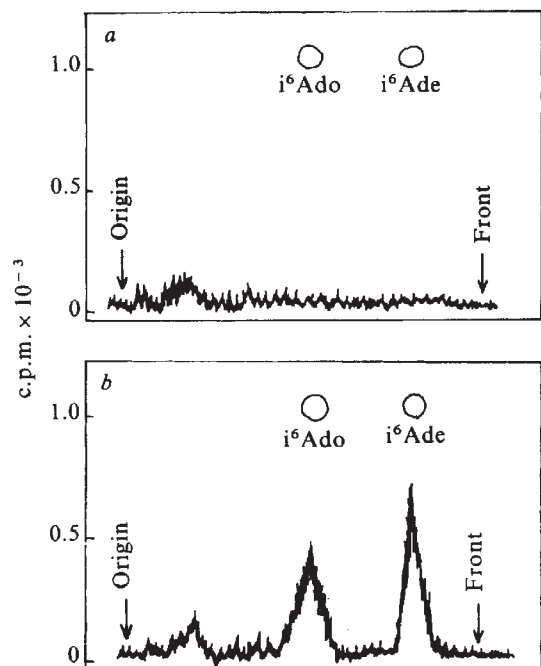
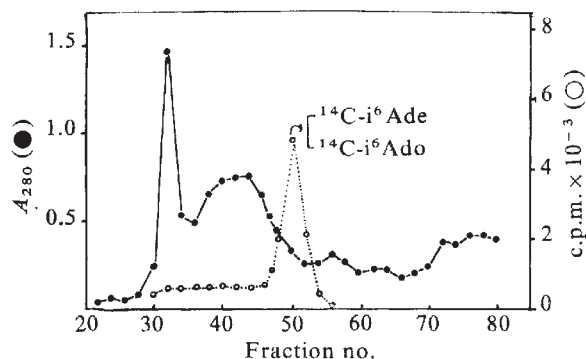


Fig. 2 Thin-layer chromatography of the products synthesised *in vitro* using ^{14}C -5'-AMP in the absence (a) or presence (b) of Δ^2 -isopentenylpyrophosphate. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 0.05 μCi of $[\text{U}-^{14}\text{C}]\text{5'-AMP}$ (538 mCi mmol^{-1} , Radiochemical Centre), 0.5 mM Δ^2 -isopentenylpyrophosphate and 0.5 mg of dialysed S-100 in a final volume of 100 μl . After 60 min incubation at 27 $^{\circ}\text{C}$, the reaction mixture was extracted with ethylacetate. The extract was subjected to thin-layer chromatography in chloroform-acetic acid (1:1, v/v) and then scanned as described in the legend to Fig. 1. Δ^2 -Isopentenylpyrophosphate was synthesised chemically by the method of Kandutsch *et al.*⁵.

These results led us to investigate the cell-free biosynthesis of $i^6\text{Ade}$ in this organism. We report here the existence of a cytokinin biosynthetic pathway not related to tRNA degradation, showing that 5'-AMP is the actual acceptor molecule for the isopentenyl group. Our findings suggest that the biosynthesis of cytokinin in higher plants may proceed in a similar fashion.

We first synthesised $i^6\text{Ade}$ in reaction mixture containing ^{14}C - Δ^2 -isopentenylpyrophosphate and crude extracts (S-100) of

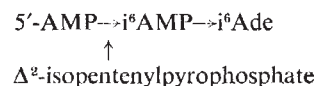
Fig. 3 Gel filtration of the S-100 extract on a Sephacryl S-200 column. S-100 extract, containing 15 mg of protein, was applied to a Sephacryl S-200 column ($1.5 \times 70 \text{ cm}$) which had previously been equilibrated with buffer B (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 6 mM 2-mercaptoethanol and 10% glycerol). Elution was carried out with buffer B and 1.5 ml fractions were collected. $i^6\text{Ade}$ synthesis was assayed with the reaction mixture described in Fig. 2, except that 20 μl of each fraction was used instead of S-100. After incubation at 27 $^{\circ}\text{C}$ for 60 min, the reaction mixture was extracted with 150 μl of ethylacetate and 30 μl of the ethylacetate layer was counted in a Beckman, Model LS-200B, liquid scintillation counter in toluene-based scintillator.



D. discoideum at the stage of late culmination. As shown in Fig. 1, dialysed S-100 catalysed the formation of a radioactive material that coincided in position with the authentic $i^6\text{Ade}$ spot only when the supernatant of nondialysed, boiled S-100 was added. Essentially similar results were obtained with another solvent system (chloroform : methanol, 8 : 2, v/v). These findings suggest that $i^6\text{Ade}$ was in fact synthesised and that a heat-stable, dialysable substance was the actual acceptor of the isopentenyl group. Tests on various compounds related to adenine showed that adenine nucleotides such as 5'-AMP, ADP, ATP and 3', 5'-cyclic AMP, but not adenine or adenosine, could replace the supernatant of nondialysed, boiled S-100 in synthesis of $i^6\text{Ade}$ (data not shown). These results were confirmed in another kind of reaction using ^{14}C -labelled compounds as acceptors and unlabelled Δ^2 -isopentenylpyrophosphate as donor; addition of 5'-AMP, ADP, ATP or cyclic AMP led to formation of $i^6\text{Ade}$, while addition of Ade or Ado did not. An example is shown in Fig. 2. In this experiment, isopentenyladenosine ($i^6\text{Ado}$) was also produced. Of the adenine nucleotides tested, 5'-AMP seemed to be the direct acceptor of isopentenyl group, because addition of unlabelled 5'-AMP strongly inhibited the formation of $i^6\text{Ade}$ when ^{14}C -ADP, ^{14}C -ATP or ^{14}C -cyclic AMP was used, whereas addition of cold ADP, ATP or cAMP only slightly inhibited the synthesis of ^{14}C - $i^6\text{Ade}$ from ^{14}C -AMP (data not shown).

To confirm the identity of the direct substrate in synthesis of $i^6\text{Ade}$, S-100 was purified by gel filtration on a Sephacryl S-200 column (Fig. 3), and each fraction was assayed for formation of a radioactive product extracted with ethylacetate, using ^{14}C -AMP and unlabelled Δ^2 -isopentenylpyrophosphate as substrates. The activity appeared in a central fraction as a sharp peak. The radioactive products were found to be a mixture of ^{14}C - $i^6\text{Ade}$ and ^{14}C - $i^6\text{Ado}$. With this fraction as enzyme source, ^{14}C -cyclic AMP or ^{14}C -ATP was not utilised for the synthesis of $i^6\text{Ade}$ and $i^6\text{Ado}$, indicating that 5'-AMP is the direct acceptor of the isopentenyl group. It is very likely that the first reaction product is isopentenyl AMP ($i^6\text{AMP}$) and that this is converted to $i^6\text{Ado}$ and $i^6\text{Ade}$. To confirm this possibility, the peak fraction in Fig. 3 was further purified by phosphocellulose column chromatography. Fractions were assayed by analysing the total reaction products by thin-layer chromatography without previous treatment of the mixture with ethylacetate. As shown in Fig. 4, ^{32}P -AMP was in fact synthesised from ^{32}P -AMP. Radioactive $i^6\text{AMP}$ was also synthesised by fractions 18 to 20 using ^{14}C -AMP instead of ^{32}P -AMP without formation of ^{14}C - $i^6\text{Ade}$ or ^{14}C - $i^6\text{Ado}$ (data not shown). These results show that the first reaction product is $i^6\text{AMP}$. Because scarcely any $i^6\text{Ade}$ or $i^6\text{Ado}$ was detected, the enzymes which convert $i^6\text{AMP}$ to these products were thought to be separated from the enzyme synthesising $i^6\text{AMP}$.

It can be concluded from these studies that the overall pathway for $i^6\text{Ade}$ synthesis is as follows:



Further investigations are needed to determine whether $i^6\text{Ade}$ is formed directly from $i^6\text{AMP}$ or through $i^6\text{Ado}$. $i^6\text{AMP}$ was scarcely detected in the reaction mixture using a crude extract, due to its quick degradation to $i^6\text{Ade}$. This indicates that the cells contain exclusively $i^6\text{Ade}$, but not its nucleotide. In this respect it should be noted that cytokinin activity of $i^6\text{Ade}$ is almost 2 orders of magnitude higher than that of $i^6\text{Ado}$, $i^6\text{AMP}$ and cyclic $i^6\text{AMP}$, when the activity was determined by the tobacco assay⁸.

It is well known that cyclic AMP acts as a chemotactic factor causing aggregation of *D. discoideum* cells^{9,10}. 5'-AMP, which is the first substrate for $i^6\text{Ade}$ biosynthesis, is formed by hydrolysis of cyclic AMP by cyclic AMP phosphodiesterase, and that cyclic AMP, $i^6\text{Ade}$ and discadenine are all compounds related to adenine. Thus these compounds may be closely inter-

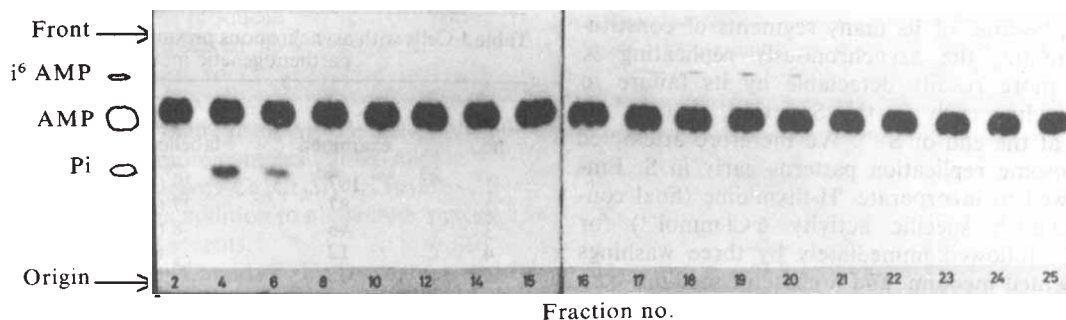


Fig. 4 Autoradiography of a thin-layer chromatogram of the products synthesised by the fractions obtained by phosphocellulose column chromatography. The peak fractions from Sephacryl S-200 (fractions 49–51) were pooled and applied to a phosphocellulose column (Whatman P11, 0.7×12 cm) which had previously been equilibrated with buffer B. Elution was carried out with a linear gradient of KCl (0–0.5 M; total volume of the gradient, 30 ml) and 1-ml fractions were collected. The reaction mixture contained 20 mM Tris-HCl pH 7.5, 5 mM magnesium acetate, 0.5 mM Δ^2 -isopentenylpyrophosphate 0.2 μ Ci of 32 P-5'-AMP (500 mCi mmol $^{-1}$) and 20 μ l of phosphocellulose column fraction in a final volume of 50 μ l. After incubation at 27 °C for 60 min, reaction mixture was cooled in ice and 5- μ l portions were spotted directly onto thin-layer plates coated with cellulose powder (20 $\times$ 20 cm, Funakoshi). Material was developed using isobutyric acid–0.5 M NH_4OH (5:3, v/v). 32 P-5'-AMP was synthesised by the method of Symons⁶. Unlabelled i^6 AMP used as marker was synthesised by the method of Yoshikawa *et al.*⁷.

related, with important roles in regulation of the development of *D. discoideum*. In *D. discoideum*, i^6 Ade is an intermediate in discadenine biosynthesis, but it probably also has some important physiological activity itself, like cytokinin in higher plants.

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X chromosome inactivation in diploid parthenogenetic mouse embryos

THE mechanism by which one X chromosome in normal cells of female mammals becomes inactive while the other, apparently identical, chromosome remains active is not known. In kangaroos the paternally derived X chromosome is preferentially inactivated in most tissues¹, and in mice and rats similar preferential inactivation of the paternal X chromosome occurs in the cells of the extraembryonic membranes^{2,3}. Paternal X inactivation in kangaroos led Cooper⁴ to postulate that passage of the X chromosome through male gametogenesis was an important factor in its inactivation. He⁴ and Brown and Chandra⁵ suggested that paternal X inactivation was a primitive form and that random X inactivation as seen in adult eutherian mammals had evolved from it. Brown and Chandra further suggested that passage of one chromosome set through male gametogenesis or fertilisation led to chromosomal imprinting. A locus concerned in the control of X chromosome activity was postulated to become inactive when imprinted, and one active copy of this gene could maintain the activity of one

X chromosome. In marsupials this gene was postulated to lie on the X chromosome itself, but on an autosome in eutherians. Thus, in eutherians the number of active X chromosomes in any animal should be equal to the number of maternally derived (and therefore non-imprinted) autosome sets. In this context it is interesting to study X inactivation in artificially formed parthenogenetic embryos, in which all chromosomes are of maternal origin. We report here that diploid parthenogenetic mouse embryos, at the late egg cylinder stage, showed a single late replicating chromosome, indicating that X inactivation had occurred normally. Because in the production of these embryos polar body formation had been suppressed, it seems that neither passage of chromosomes through male gametogenesis, nor fertilisation, nor probably the effects of peripheral egg cytoplasm are required for normal X inactivation in the mouse.

In the normal mouse embryo X chromosome inactivation is thought to occur at the late blastocyst stage⁶, and a late replicating X chromosome can be recognised at about the 5-d⁷ to 7-d stage⁸. Thus, studies of DNA replication in parthenogenetic embryos at the late egg cylinder stage should indicate whether normal X inactivation has occurred.

Experimental parthenogenetic mouse embryos have been produced by various means⁹, and Kaufman *et al.*¹⁰ have achieved development of a high proportion of diploid parthenogenotes to the egg cylinder stage or later. We have now studied DNA replication by ^3H -thymidine labelling at the late egg cylinder stage in diploid parthenogenetic embryos produced by their method. Eggs were activated by culturing cumulus masses containing recently ovulated oocytes for 5–6 h in medium lacking calcium and magnesium salts. Then the cumulus cells were removed with hyaluronidase, and the successfully activated presumptive diploid eggs (which developed two pronuclei after suppression of second polar body formation or extrusion) were cultured for a further 90 h in normal embryo culture medium, by which time approximately 60% had reached the blastocyst stage. Blastocysts were then transferred to pseudopregnant recipient females, which had been mated to proven sterile vasectomised males 2.5 d earlier. Recipients were ovariectomised bilaterally at the time of transfer of blastocysts, and pregnancy was maintained by exogenous hormones, so that the time of implantation could be controlled. Implantation began about 24 h after the first of a series of oestradiol injections, which were started 6 d after blastocyst transfer. Females were killed after 4 d of injections when the embryos were expected to be at a stage equivalent to about day 7.5–8.5 of normal development. The embryos were dissected free from the decidua and embryonic membranes, and placed in Eagle's medium, supplemented with foetal calf serum (20%) at 37 °C.

In the mouse, because of its many segments of constitutive heterochromatin, the asynchronously replicating X chromosome is more readily detectable by its failure to take up ^3H -thymidine early in the S period, than by its excess labelling at the end of $\text{S}^{8,11}$. We therefore attempted to study chromosome replication patterns early in S. Embryos were allowed to incorporate ^3H -thymidine (final concentration $5 \mu\text{Ci ml}^{-1}$; specific activity 6 Ci mmol^{-1}) for 30 min at 37°C , followed immediately by three washings with unsupplemented medium, and a chase of medium containing a 100-fold excess of unlabelled thymidine for 10 h. Colchicine, at a final concentration of $1 \mu\text{g ml}^{-1}$, was present during the final 2 h of the 10-h incubation. Air-dried slides were prepared according to a modification of the method of Wroblewska and Dyban¹² and autoradiographs were prepared.

Of six embryos studied four yielded scorable mitoses. Cells with 40 chromosomes were scored for uptake of label, and for presence or absence of a single large unlabelled chromosome, presumed to be an X chromosome. In all four embryos some cells showed a clear asynchronously replicating chromosome (Table 1). In all cells this was a large chromosome, of approximately the expected size of an X chromosome (Fig. 1). In most cases the asynchronous chromosome was unlabelled in an otherwise fully labelled cell (Fig. 1a, b), but in a few cells there was a single heavily labelled chromosome in a lightly labelled set (Fig. 1c). In addition some interphase cells showed an apparent X chromatin body (Fig. 1d).

The proportion of cells showing any label varied somewhat (from 17 to 42%) among the embryos, but the proportion of labelled cells with an asynchronous chromosome

Table 1 Cells with asynchronous presumed X chromosomes in diploid parthenogenetic mouse embryos

Embryo no.	Cells examined	Cells labelled (%)	Labelled cells with asynchronous chromosome (%)
1	107	39 (36)	25* (64)
2	87	27 (31)	15* (56)
3	46	8 (17)	5 (63)
4	12	5 (42)	4 (80)
Total	252	79 (31)	49 (62)

* A few cells (four in embryo 1 and two in embryo 2) showed a late replication pattern, that is a single heavily labelled chromosome in an otherwise lightly labelled cell.

showed reasonable consistency, with a mean of 62% (range 56–80%). The failure to detect an asynchronous chromosome in the remaining cells does not of course mean that one was lacking, because the cell cycle times may have been variable, with some cells being at mid-S, and therefore unsuitable for showing asynchronous replication, at the time of the ^3H -thymidine pulse. The finding of a few cells with a replication pattern characteristic of late S emphasises this point.

Our interpretation is thus that the asynchronously replicating chromosome seen in all four embryos was a late replicating (and therefore inactive) X chromosome. Clearly differentiation of two X chromosomes within a cell can take place in embryonic development without the need for fertilisation or for passage of either chromosome, or indeed any autosome, through male gametogenesis.

A similar conclusion was reached by McCaw and Latt¹³ who found late replicating X chromosomes in human

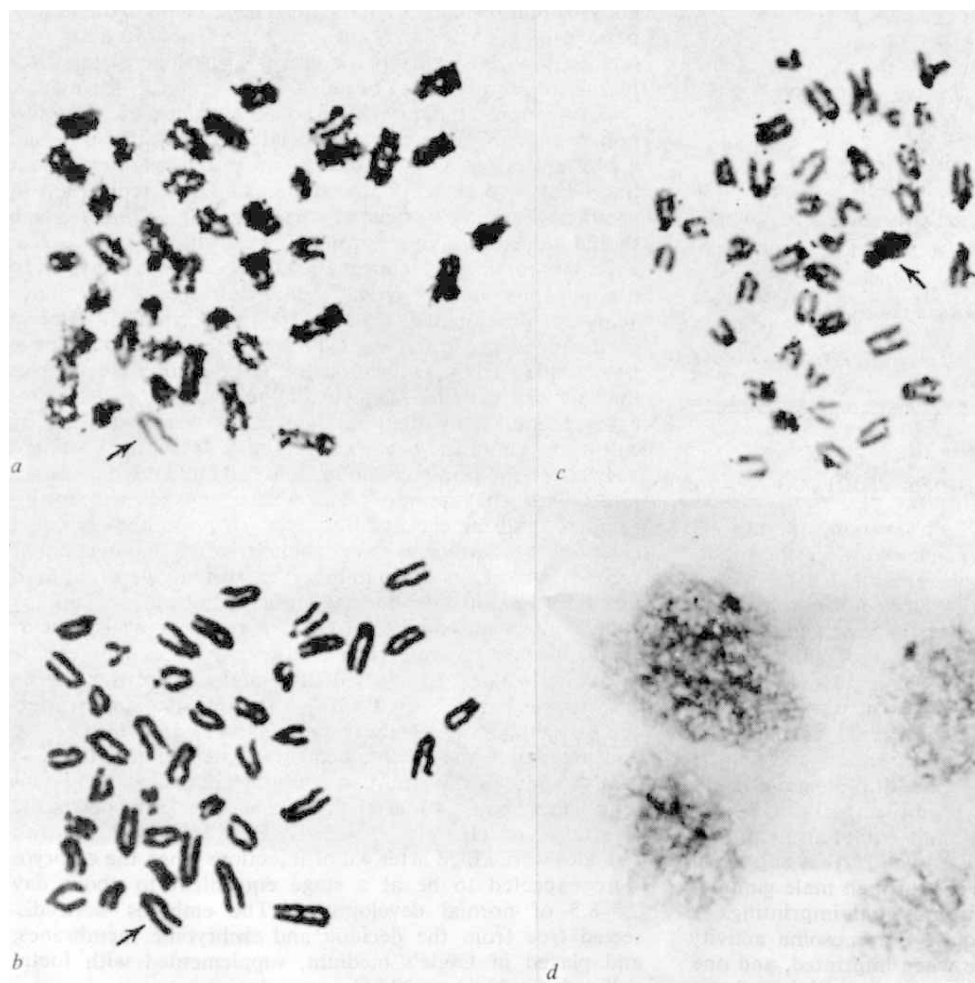


Fig. 1 a–c, Autoradiographs of cells from diploid parthenogenetic mouse embryos. a, Single unlabelled chromosome in heavily labelled cell. b, Same cell after removal of silver grains. c, Single heavily labelled chromosome in otherwise lightly labelled cell (characteristic late-S replication pattern). d, Apparent X chromatin body in some interphase nuclei. Ilford Nuclear Research emulsion, gel form L4, was used for autoradiography. Slides were exposed for 3 weeks at room temperature, developed in Amidol developer, fixed and stained with Giemsa. Silver grains were removed by transferring slides through xylene and graded alcohols to potassium ferricyanide, sodium thiosulphate and distilled water.

ovarian teratomata, which arise by parthenogenetic growth of germ cells¹⁴, and by Linder and Power¹⁴ on the basis of X-linked gene expression in such teratomata. Our work strengthens the finding by dealing with embryos which were undergoing fairly normal development (up to the relevant stage), rather than teratomata in which the genetic programming might be in some way disturbed. Thus, these results are inconsistent with the expectation from Brown and Chandra's hypothesis that the number of active X chromosomes in any cell should be equal to the number of maternally derived autosome sets. In their development¹⁵ of the original hypothesis they reconciled the X inactivation seen in certain human ovarian teratomata and a diploid/triploid mosaic by postulating that chromosomal imprinting occurred not during male gametogenesis but during passage of chromosomes through the egg cytoplasm. Normally this would occur at fertilisation, but in the cases mentioned the effect was produced by re-entry of the polar body. Our embryos were inconsistent even with this, because the parthenogenetic activation was produced in combination with suppression of polar body formation. Further evidence against Chandra and Brown's ideas has come from human triploid foetuses one of which had two active X chromosomes although only a single autosome set from the mother¹⁶ and another which had a single active X chromosome but two haploid sets from the mother¹⁷.

Thus we conclude that in eutherian mammals there is no evidence that chromosomal imprinting is required for the initiation of X-chromosome inactivation. This does not, of course, exclude the possibility of imprinting in normal embryogenesis. Preferential inactivation of the paternally derived X chromosome (manifested by late replication as detected by acridine orange fluorescence after BUdR incorporation) has been found in the extraembryonic membranes but not the foetus itself of both the mouse and the rat^{2,3}. Thus it seems probable that in eutherians, as in marsupials, imprinting of the paternally derived X chromosome does occur, but that the cells of the embryo proper escape from imprinting before X inactivation¹⁸. One might expect therefore that in parthenogenetic embryos X inactivation would be in some way abnormal in the extraembryonic membranes. Further work is necessary to test this point.

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Localisation and stimulation of prostacyclin production in vascular cells

PROSTACYCLIN (PGI₂) was reported by Vane and co-workers as a novel short-lived metabolite of prostaglandin endoperoxides (PGG₂ and PGH₂) which inhibited platelet aggregation¹. Subsequent studies have demonstrated that PGI₂ is the most potent inhibitor of platelet aggregation so far described, acting by stimulating platelet adenylate cyclase^{2–4}. PGI₂ is produced by isolated blood vessel segments⁵ and is therefore likely to be of great importance in the maintenance of vascular homeostasis. The cellular localisation and regulation of PGI₂ synthesis have not previously been established, although it has been proposed that the main source of PGI₂ is through pro-aggregatory prostaglandin endoperoxides released from platelets being enzymatically converted to PGI₂ by vascular endothelial cells^{5,6}. We show here that pig aortic endothelial cells, but not aortic smooth muscle cells or fibroblasts, synthesise PGI₂. This production of PGI₂ is stimulated by incubating endothelial cells with PGG₂ or with its precursor, arachidonic acid. Furthermore, PGI₂ synthesis is powerfully stimulated by cell-free plasma.

Pig aortic endothelial cells, aortic medial smooth muscle cells, and aortic adventitial fibroblasts, were isolated and grown in homogeneous monolayer cultures according to previously described methods^{7,8}. For experiments cells were detached (0.1% trypsin + 0.025% EDTA; 37 °C; 2 min) and suspended in serum-free HEPES-buffered Dulbecco's medium. Subsamples were resuspended in small volumes of calcium and magnesium-free phosphate-buffered saline (PBS) immediately before use. Platelet-rich plasma (PRP) was prepared by differential centrifugation of whole blood, anticoagulated with citrate or heparin; platelet aggregation was measured photometrically⁹ in 0.1 ml samples of human PRP¹⁰. All incubations were at 37 °C. Arachidonic acid (Sigma, Grade I) was prepared as the sodium salt, stored under nitrogen at –20 °C, and used within 2 weeks. Arachidonic acid was converted enzymatically¹¹ to 15-hydroperoxyarachidonic acid (15HPAA), a specific inhibitor of PGI₂ synthesis¹², and used immediately.

In previous studies of the effects of freshly isolated endothelium on platelets¹³ (and in preliminary studies with cultured vascular cells¹⁴) observations were first made by mixing cells with PRP, and we therefore performed similar experiments. Suspensions of 1.6–3.3 × 10⁴ endothelial cells, mixed with 0.1 ml human PRP, inhibited (with similar potency) platelet aggregation induced by ADP, collagen, vasopressin or the PGG₂ analogue (15S)-hydroxy-9α, 11α-epoxymethanoprost-5Z, 13E-dienoic acid (U44069). This inhibitory effect was reduced by pre-incubating the endothelial cells (5 min) with 1 mM aspirin, an inhibitor of the cyclo-oxygenase component of the prostaglandin biosynthetic pathway¹⁵, and abolished by pre-incubating the cells with 1 mM 15HPAA. It was also reduced if PRP was pre-incubated (2 min) with 100 μM 2',5'-dideoxyadenosine (DDA), an inhibitor of platelet adenylate cyclase¹⁶. Thus, the inhibitory factor produced by mixing PRP with endothelial cells has properties characteristic of PGI₂.

In contrast, suspensions of either smooth muscle cells or fibroblasts, when mixed with PRP, induced platelet aggregation directly; the aggregation responses were similar to those produced by purified collagen, and were inhibited either by pre-incubating the vascular cells with collagenase or by pre-treating the PRP with aspirin or with a stimulant of adenylate cyclase (see Fig. 1). These results indicate that platelet aggregation was induced by cell-associated collagens known to be synthesised by these cells in culture^{17,18}. Because smooth muscle cells and fibroblasts aggregated platelets we could not, by direct mixing experiments, exclude the possibility that these cells might produce PGI₂.

Vascular tissue incubated with buffer alone, or with arachidonic acid or PGG₂, has been shown to produce sufficient PGI₂ for subsamples of the supernatant to inhibit platelet aggregation¹⁹.

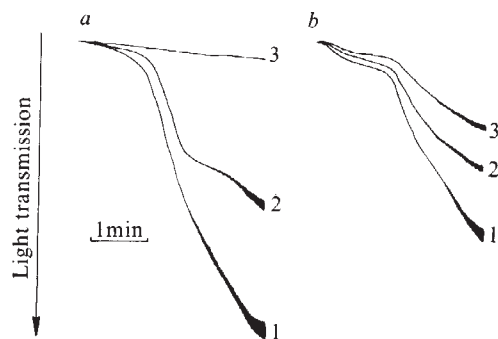


Fig. 1 Platelet aggregation induced by aortic smooth muscle cells or fibroblasts. *a*, Smooth muscle cells (5.2×10^4) in PBS were centrifuged (15,000g, 10 s) in an aggregometer cuvette and the supernatant was discarded. Human citrated PRP, 0.1 ml, was added, the mixture stirred at 37 °C and aggregation recorded. 1, Control response. 2, Cells pretreated with 0.2% collagenase in Dulbecco's medium (37 °C; 15 min). 3, PRP pretreated with 10 μ M PGD₂ (37 °C; 2 min). *b*, Fibroblasts (6.6×10^4) prepared as above. 1, Control response. 2, Cells pretreated with collagenase. 3, PRP pretreated with 1 mM aspirin (37 °C; 5 min).

We therefore adopted this approach to characterise in more detail the localisation and regulation of PGI₂ production by vascular cells. To test for spontaneous production of PGI₂, cells were incubated in PBS for 2 min and subsamples of the supernatant added to PRP. To test for stimulation of PGI₂ production, cells were pre-incubated with 1 mM arachidonic acid or 0.2 μ M PGG₂, washed in PBS alone for 2 min, and subsamples of these supernatants were tested as before. We have so far investigated PGI₂ production in aortic endothelial cell cultures, tested at between 2 and 17 passages in culture, derived from nine animals. Cells (5×10^3 – 10^5) cultured from five animals produced detectable levels of PGI₂ after incubation in PBS alone for 2 min, whereas incubations of up to 10^5 cells from the other four animals did not. We have also found PGI₂ production by endothelial cells cultured from the pig post-caval vein.

The inhibitor was characterised as PGI₂ by the following criteria: its activity was labile (half life ~ 5 min at 37 °C); its production was inhibited by pre-incubating endothelial cells with 15HPAA, aspirin, indomethacin, or mepacrine (an agent that blocks prostaglandin production in platelets by inhibiting

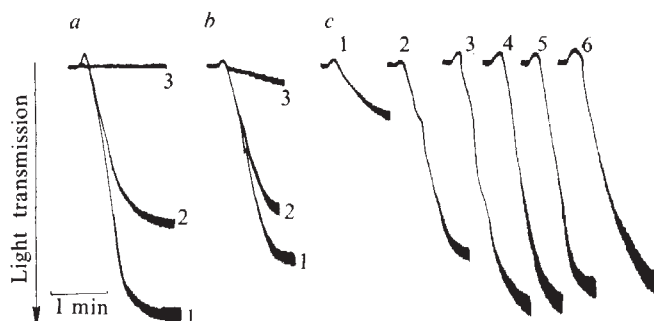
the plasmalemmal phospholipase A₂ responsible for endogenous arachidonic acid release²⁰); its effect was potentiated by pre-treating PRP with papaverine (a phosphodiesterase inhibitor) and inhibited by pretreating PRP with DDA (see Fig. 2). The inhibitor was active against primary aggregation induced by ADP, vasopressin and U44069; the effects of the latter two agonists are independent of ADP²¹, suggesting that in these conditions little, or none, of the inhibition is due to ADPase activity known to be present in aortic tissue^{22,23}. Secondary aggregation responses induced by ADP, U44069, collagen and arachidonic acid were also inhibited.

Cells from all animals tested produced easily detectable amounts of PGI₂ when stimulated with arachidonic acid or PGG₂. Although one culture, when retested after 16 passages, had lost the ability to produce detectable quantities of PGI₂, whether cells were stimulated with PGG₂ or added directly to PRP, other cells still produced PGI₂ after at least 17 passages. We have been unable to detect any enhanced PGI₂ production by pre-incubating endothelial cells (5 min) with 10–100 μ g ml⁻¹ bradykinin or 10 μ g ml⁻¹ angiotensin II, which stimulate production of prostaglandins E and F by cultured endothelium^{24,25}, and we have not observed any stimulation of PGI₂ production by thrombin at concentrations up to 25 U ml⁻¹. We have never observed the production of any inhibitor of platelet aggregation by smooth muscle cells or fibroblasts, even after stimulation with arachidonic acid or PGG₂.

Our results demonstrate that vascular endothelium, but not smooth muscle cells or fibroblasts, can synthesise PGI₂; this confirms and extends the preliminary observations made by Harker *et al.*¹⁴. Endothelial cultures from all animals tested, including those cells which failed to produce detectable PGI₂ on incubation with PBS alone, did inhibit platelet aggregation by producing PGI₂ when directly mixed with PRP. This implies that a factor (or factors) in PRP stimulate(s) PGI₂ production. One such factor could be a prostaglandin endoperoxide released from platelets; however, 5 min pre-incubation of PRP with 1 mM aspirin or 100 μ M indomethacin had no effect on the inhibition by endothelial cells of primary platelet aggregation, which indicates that platelets are not necessary for the supply of precursor prostaglandin endoperoxides to endothelial cells for PGI₂ production. To investigate the possibility that other platelet-derived products might be responsible for stimulating PGI₂ production, we tested the effects on vascular cells of cell-free plasma, prepared in conditions which minimised secretion of platelet constituents. Incubation of 3.3×10^4 endothelial cells with 0.1 ml of cell-free plasma for 2 min, then centrifuging and resuspending in PBS alone for 2 min, resulted in the production of levels of PGI₂ (characterised by the criteria used above) detectable in as little as 2 μ l of supernatant, and the amount of PGI₂ synthesised was directly related to the volume of cell-free plasma used as a stimulus (see Fig. 3). We have been able to stimulate PGI₂ production using plasma diluted as much as 50-fold with PBS. Smooth muscle cells (up to 3.3×10^5) did not produce PGI₂ on incubation with plasma. The stimulation of endothelial PGI₂ production was evident using human plasma prepared with citrate, heparin or EDTA, or human serum; and also with pig or rat plasma. PGI₂ production was stimulated equipotently by PRP or cell-free plasma, further indicating that in normal conditions platelet prostaglandin endoperoxides do not contribute significantly to endothelial PGI₂ production. The factor in cell-free plasma was heat labile (56 °C; 30 min), non-dialysable, and was still present after freezing of plasma.

The stimulation of PGI₂ production obtained using human cell-free plasma (which contains about 10 μ M arachidonic acid²⁶) was comparable to that obtained by adding 500 μ M arachidonic acid in PBS, even in the absence of albumin. It is therefore unlikely that PGI₂ synthesis is stimulated by precursor arachidonic acid carried on circulating plasma albumin. A more probable explanation is that the plasma factor stimulates PGI₂ production in a manner analogous to that by which RCS-RF (rabbit aorta contracting substance-releasing factor) stimulates

Fig. 2 Characterisation of PGI₂ production by endothelial cells. Suspensions of cells in PBS were incubated for 2 min at 37 °C centrifuged (15,000g, 10 s) and subsamples of the supernatant (50 μ l, or 50 μ l of PBS alone for controls) added to 50 μ l human citrated PRP. After mixing for 1 min at 37 °C, aggregation responses to 1 μ M ADP were measured. *a*, 1, Control; 2, supernatant from 1.6×10^4 cells; 3, supernatant from 6.6×10^4 cells. *b*, 1, Supernatant from 6.6×10^3 cells; 2, control, but PRP pre-incubated (37 °C, 2 min) with 20 μ M papaverine; 3, as 1 but PRP pre-incubated with papaverine. *c*, 1, Supernatant from 3.3×10^4 cells; 2–6, as 1, but: 2, PRP pre-incubated (37 °C 2 min) with 100 μ M DDA; 3, cells pre-incubated (37 °C, 5 min) with 1 mM 15HPAA in PBS (before incubating in PBS alone); 4, cells pre-incubated (37 °C, 5 min) with 1 mM aspirin; 5, cells pre-incubated (37 °C, 5 min) with 100 μ M indomethacin; 6, cells pre-incubated (37 °C, 5 min) with 50 μ M mepacrine.



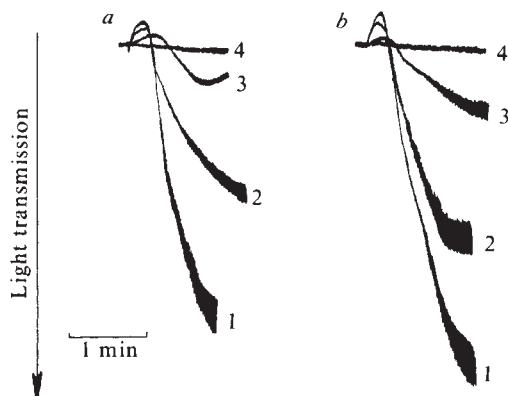


Fig. 3 Stimulation of endothelial cell PGI_2 production by cell-free plasma. Suspensions of 3.3×10^4 cells were pre-incubated in 0.1 ml human citrated cell-free plasma, or plasma diluted with PBS, for 2 min (37°C); centrifuged ($15,000g$, 10s), re-suspended in 0.1 ml PBS and incubated for a further 2 min. After centrifuging, subsamples of the supernatant were added to 50 μl of human citrated PRP, with sufficient PBS to give a final volume of 0.1 ml. After mixing for 1 min at 37°C , aggregation responses to $1 \mu\text{M}$ ADP were measured. *a*, Cells treated with undiluted plasma. 1, Control, 50 μl PBS alone; 2, 2 μl supernatant; 3, 10 μl supernatant; 4, 25 μl supernatant. *b*, 1, control; 2, 50 μl supernatant, but cells pretreated with 0.1 ml plasma diluted 1:10 with PBS; 3, as (2) but plasma diluted 1:1; 4, as (2) but plasma undiluted.

thromboxane A_2 production in lung tissue²⁷. This suggestion is strengthened by our finding that 5 min pre-incubation of endothelial cells with $4 \mu\text{g ml}^{-1}$ betamethasone, which antagonises RCS-RF activity²⁷, blocked PGI_2 synthesis. The lack of detectable effects of bradykinin and angiotensin II on PGI_2 synthesis, in contrast to their stimulation of endothelial PGE and PGF production^{24,25}, suggests that the plasma factor may selectively stimulate PGI_2 production.

The previous proposal that vascular PGI_2 production may be an important regulatory mechanism in haemostasis and thrombosis^{1,5} is reinforced by our findings that PGI_2 synthesis is localised in endothelial cells and that cell-free plasma alone stimulates PGI_2 production.

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Note added in proof: Since this manuscript was submitted, three directly relevant papers have been published. Weksler *et al.*²⁸ present chemical evidence for prostacyclin production by cultured human and bovine aortic endothelium, but not by human fibroblasts. Baenziger *et al.*²⁹ report the synthesis of prostacyclin by cultured human aortic smooth muscle cells and fibroblasts. We therefore carried out further experiments, and by using $>3.3 \times 10^5$ smooth muscle cells, have found some strains to produce a transferable inhibitor of platelet aggregation. To produce comparable inhibition, however, required approximately $100 \times$ smooth muscle cells as endothelial cells. Moncada *et al.*³⁰ have shown that the conversion of PGH_2 to prostacyclin by rabbit aorta is at least tenfold greater (on a wet weight basis) in the intimal layer than in the media or adventitia.

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Sister chromatid exchange as an indicator of mutagenesis

SISTER chromatid exchange (SCE), that is, the reciprocal interchange of DNA between chromatids, is easily visualised in metaphase chromosomes^{1,2} and has been applied to study chromosome structure^{3,4}, chromosome damage⁵, and instability and DNA repair deficiency syndromes^{6–9}. Since SCEs can be induced by subtoxic doses of carcinogens and mutagens^{5,10–13}, their analysis offers the possibility of a rapid, sensitive and quantitative assay for genetic damage. We have begun to examine the relation between SCEs and mutations in Chinese hamster ovary (CHO) cells by quantifying the induction of SCEs in parallel with the induction of mutations producing 8-azaguanine resistance, that is, mutations predominately at the hypoxanthine phosphoribosyltransferase, *hprt*, locus. Since the conversion of a chemically induced DNA lesion to a SCE or mutation may depend on the nature of that lesion, we tested four chemicals that differ in their interaction with DNA—ethyl methanesulphonate (EMS; O^6 : N^7 guanyl alkylation ratio¹⁴ of 0.03), *N*-ethyl-*N*-nitrosourea (ENU; O^6 : N^7 guanyl alkylation ratio¹⁵ of about 0.35), the crosslinking agent mitomycin C (MMC)¹⁶ and the intercalator proflavine sulphate (PRO)¹⁷. Our results indicate a linear relation between induced SCEs and mutations. The relative efficiency, however, is different for each chemical.

As shown in Fig. 1, EMS, ENU and MMC produced a linear increase in both induced SCEs and mutations as a function of dose. All six regression lines for these chemicals had slopes significantly different from zero. Proflavine did not produce a significant increase ($P>0.05$) in either SCEs or mutations over the dose range tested, even though the highest dose (1.6 μM) reduced cell survival to approximately 60%. (Note the range of the ordinate in Fig. 1d compared to the other chemicals). For comparison with the other chemicals, however, we have treated these data as an indication of a positive response in both assays. Each of the four

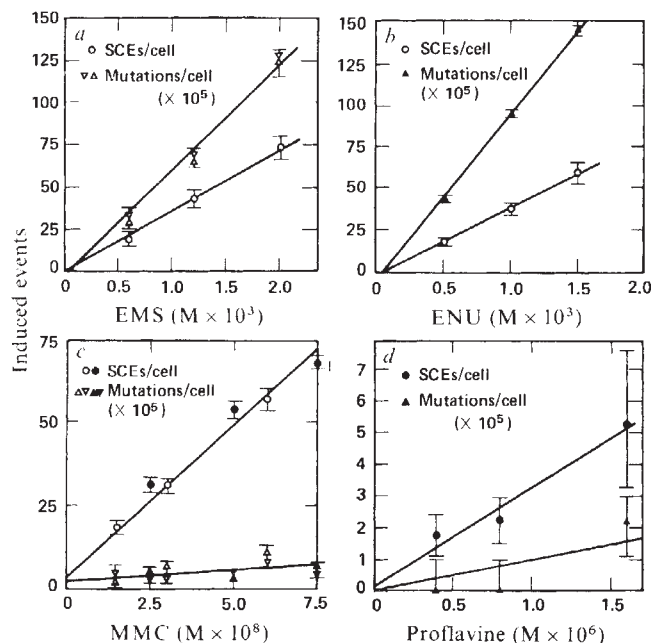


Fig. 1 The induction of SCEs and mutations as a function of dose for: a, EMS; b, ENU; c, MMC; and d, PRO. Circles represent SCEs per cell; triangles represent mutations per cell $\times 10^5$. A suspension-adapted clone of the CHO line designated SC1 was cultured in 200-ml spinner flasks in a minimum essential medium (MEM) with 10% foetal calf serum. With the cells growing exponentially at a density of $1.3 \times 10^5 \text{ ml}^{-1}$, 5-bromodeoxyuridine (BrdU), at a final concentration of $10 \mu\text{M}$, and the test chemicals were added to each culture. Control cultures contained only BrdU. After 18 h exposure (approximately 1.5 generations), the cells were collected by centrifugation, then rinsed once and resuspended in fresh medium. At each dose the culture was divided into two portions. The first fraction of each treated culture was subdivided into four identical 20-ml roller-tube cultures containing $10 \mu\text{M}$ BrdU, which were used to prepare chromosomes for SCE analysis. To ensure collection of the majority of second-division cells, sequential cell collection of the roller tubes was performed at 4-h intervals from 18 to 34 h. Colcemid (10^{-7} M final concentration) was added 4 h before cell collection. SCEs were differentiated by the FPG technique³ and 25 cells were scored at each dose. For each 4-h collection interval an average SCE frequency for each dose was calculated by weighting the SCE frequency by the mitotic index of second-division cells in that interval. All operations before cell fixation or plating for mutations were performed in the dark or under a safelight. The second fraction of each treated culture was grown further, with low-ratio dilutions, in the absence of BrdU for mutation expression and selection. On days 4 and 5 (EMS and MMC) or day 4 (ENU and PRO) after removal of the chemicals these cultures were rinsed with serum-free medium and plated into regular medium (α -MEM + 10% dialysed foetal calf serum) for plating efficiency determinations, and into the same medium containing $15 \mu\text{g ml}^{-1}$ 8-azaguanine for selection of drug-resistant mutant colonies. The use of dialysed serum produced a reproducible assay in which all colonies isolated and tested were more drug-resistant than wild type¹⁸. The frequency of mutant colonies detected was proportional to cell inoculum in the conditions used, showing that cell density was not a complicating factor. Ten replicate 100-mm Petri dishes were seeded with 4×10^5 cells each and stained after 14 d of incubation. Plating efficiencies were determined by inoculating 300 cells per dish (five replicates) and staining after 8 d. Mutation expression was found to be maximal by four generations after exposure. The average mutation frequency in the presence of BrdU alone (controls) was $5.18 \pm 0.65 \times 10^{-5}$ ($\pm \text{s.e.m.}$) mutants per survivor. There was no apparent increase in the spontaneous mutation frequency from the presence of BrdU. The frequency of SCE in the controls was 7.03 ± 0.24 per cell averaged over five experiments. Control values were subtracted to obtain induced frequencies. The data were fit using a least squares linear regression with 0,0 as a data point. The open and closed triangles for MMC in (c) indicate two independent experiments. The two triangles for each dose in (a) and (c) represent day 4 and 5 platings; data were pooled for the regression analysis. Standard errors of the mean for the SCE data represent the sample time variation for each dose. Standard errors for the mutation frequencies represent Poisson counting error.

chemicals induced both SCEs and mutations over the same concentration range, suggesting a similarity in the sensitivity of the two assays. The slope of each regression line from Fig. 1 was used as a measure of the induction efficiency for SCEs or mutations, as shown in Table 1. On a molarity basis, both SCEs and mutations increase over four orders of magnitude as follows: EMS < ENU < PRO < MMC.

In Fig. 2 the frequency of induced SCEs per cell is compared with the frequency of induced mutations. The data show a linear relation between SCEs and mutations, but the ratio of induced SCEs to induced mutations (regression line slope) is different for each chemical (Table 1). Of the four chemicals, ENU is the least efficient inducer of SCEs compared with mutations (lowest ratio) while MMC is most efficient (highest ratio); these compounds differ by a factor of 15. Thus, it is clear that there is a positive, linear correlation between SCEs and mutations, but the relative efficiency of SCE and mutation induction varies with the compound. The type of lesion produced by each chemical and the nature of its repair probably determine the relative frequency of induced SCEs and mutations. Therefore, in order for SCEs to be a quantitative predictor of mutation induction, calibration curves similar to those in Fig. 2 may have to be derived for each agent.

Obviously many more chemicals must be compared before any general conclusions can be drawn concerning the relation of SCEs and mutations. Also, other genetic markers must be tested since azaguanine resistance may not have typical mutagen sensitivity for each chemical. If we do assume that this marker yields a representative mutation rate and that the ratio of SCEs to mutations (Table 1) is similar in human cells, then for the approximately 50,000 structural genes in man¹⁹, the above data suggest that for every SCE per cell: EMS produces 0.85 mutations per cell; ENU produces 1.2 mutations; PRO produces 0.28 mutations; and MMC produces 0.08 mutations. The use of this rapid cytological SCE analysis as a quantitative indicator of mutagenesis seems feasible but must be approached with caution since each chemical or class of compounds may respond differently. The SCE:mutation ratio may also differ with species, tissue type, dose rate, or other factors, making it necessary to establish the relationship for the exposure condition of interest. If an SCE:mutation ratio obtained *in vitro* can be extrapolated to cells *in vivo*, it will then be possible to predict mutation rates in man from SCE

Fig. 2 The relationship between induced SCEs and induced mutations. Each set of data was fitted by a least squares linear regression with 0,0 as a data point. For MMC, the two sets of solid triangles represent separate dose responses performed on different days; the two sets were combined to generate a single regression line.

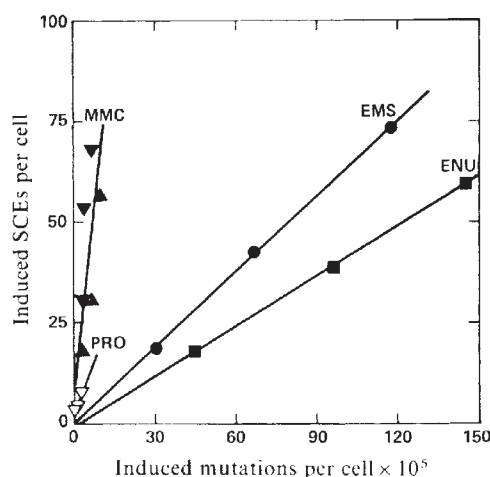


Table 1 Efficiency of induction of SCEs and mutation to azaguanine-resistance

Chemical	SCE per cell per mol per l		mutations per cell per mol per l		SCE per mutation	
	Absolute* ($\times 10^{-6}$)	Relative	Absolute* ($\times 1$)	Relative	Absolute† ($\times 10^{-4}$)	Relative
EMS	0.037	1.0	0.62	1.0	5.9	1.0
ENU	0.040	1.1	0.97	1.56	4.1	0.70
PRO	3.2	86	14.1	22.7	18	3
MMC	918	2.5×10^4	914	1.5×10^3	60	10

*Absolute efficiency is the slope of each dose response regression line in Fig. 1.

†Absolute ratio taken from slope of the regression lines in Fig. 2.

data derived from human cells exposed *in vivo* or *in vitro*.

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Eosinophil chemotactic factor release from neutrophils by *Nippostrongylus brasiliensis* larvae

INFESTATIONS with metazoan parasites are the most efficient stimulus known for the induction of eosinophilia, and eosinophils have been shown to have an essential role in the elimination of worms^{1,2}. Eosinophil chemotactic factors have been derived from lymphocytes³⁻⁵, mast cells⁶ and the complement system⁷. We reported recently the *in vitro* release of a low molecular weight eosinophil chemotactic factor, ECF, from human neutrophils (PMN) during phagocytosis of zymosan⁸. The evidence presented here shows that neutrophils, after incubation with larvae of *Nippostrongylus brasiliensis* (N.B.), can also generate this same factor which selectively attracts eosinophils *in vitro*. As neutrophils are the first cells to accumulate at tissue sites where parasites are located⁹⁻¹¹, the attraction of eosinophils may present an early line of defence against the invading organism.

N. brasiliensis-infested Wistar rats^{12,13} were used as a model system as it is difficult to obtain sufficient numbers of organisms from infected humans. Larvae were obtained from the faeces of infested Wistar rats, washed twice in a Tris-albumin buffer containing calcium and magnesium¹⁴, and incubated with 1×10^7 purified human PMN in a total of 1 ml at 37 °C for the time indicated. PMN were separated from other peripheral leukocytes by Ficoll-Hypaque¹⁵ flotation, followed by dextran sedimentation to eliminate erythrocytes. Cells and larvae were removed by rapid centrifugation in the cold and the supernatants analysed for eosinophil chemotactic activity using the method described previously¹⁴. Results are expressed as the

number of eosinophils that have migrated completely through nitrocellulose filters (Sartorius) in five 10×40 microscopic fields. Human and guinea pig eosinophils used in these studies gave comparable results. Controls with buffer alone were always negative. This assay of chemotaxis does not measure the weak eosinophil chemotactic activity induced by very low histamine concentration¹⁴.

Table 1 shows the release of ECF with varying numbers of larvae after a 20-min incubation. Neither larvae nor cells alone induced eosinophil migration, but the mixture of both caused a dose-dependent release of eosinophil chemotactic activity. Similar results were obtained with normal human PMN, with normal rat peritoneal cells or with cells from rats infected with larvae 10 days earlier. The slight increase in histamine release (Table 1) was not detectable in other experiments, indicating that the PMN and not the mast cells were the source of the ECF. Similarly, histamine levels were not raised in the PMN supernatants, excluding the rather unlikely possibility of release of ECF from unsensitized basophils. Chromatography of either active cell supernatants on a Sephadex G-25 column showed that, in each case, the eosinophil chemotactic activity was eluted in the same fractions as the ECF obtained from basophils by anti-IgE (ref. 14) and from PMN on exposure to zymosan (molecular weight about 500) (ref. 16). The differently induced ECF preparations did not differ in their biological behaviour such as the preferential attraction of eosinophils¹⁶.

The time course of ECF-release from PMN was also studied (Table 2). After a lag period of 5 min, there was rapid release of the mediator for up to 20 min and then a gradual decline. A very similar curve can be obtained during ECF release from PMN with zymosan¹⁶. The decreasing activity after extended incubation has been shown to be due to an inactivator released together with ECF from PMN¹⁷. The lysosomal enzyme β -glucuronidase (measured by the method of Brittinger¹⁸) was released in a time dependent fashion with no decline in release rate at later time intervals.

Opsonisation of the larvae enhanced release of both ECF and β -glucuronidase from the cells. The totally passive role of the larvae in these processes was underlined by the fact that immitile parasites which had been stored in the frozen state for several weeks were just as efficient in stimulating the cells as

Table 1 Release of ECF and histamine from human PMN and rat peritoneal cells

Larvae (ml ⁻¹)	No. cells Eos/5 HPF	PMN 1×10^7 /ml		Rat peritoneal cells 1×10^7 /ml	
		Eos/5 HPF	% Histamine	Eos/5 HPF	% Histamine
0	0±0	0-0	1.0	0±0	2.0
8	0±0	0±0	1.0	15±2	3.0
80	0±0	32±4	0.5	41±4	5.0
800	0±0	109±7	1.0	279±12	6.0

ECF release was measured after 20 min incubation at 37 °C. Eos/5 HPF, eosinophils that have migrated through the filter in five high-power fields. Human PMN, 3% eosinophils, 6% lymphocytes, 0.2% basophils, 91% neutrophils. Rat peritoneal cells, 5% mast cells, 13% eosinophils, 7% neutrophils, 75% lymphocytes and macrophages.

Table 2 Release of ECF (Eos/5 HPF) and β -glucuronidase¹⁸ from 1×10^7 purified human PMN (> 90% neutrophils) incubated with 80 larvae in 1 ml buffer

Time (min)	ECF (Eos/5 HPF)	β -Glucuronidase (A_{540})
0	0 $\pm$ 0	0.017
5	0 $\pm$ 0	0.025
10	30 $\pm$ 3	0.067
15	38 $\pm$ 4	0.077
20	41 $\pm$ 3	0.078
30	24 $\pm$ 2	0.078
60	20 $\pm$ 2	0.099

ECF and β -glucuronidase release was measured after incubation at 37 °C for the times shown.

fresh larvae.

The interaction of larvae and neutrophils was followed under the light microscope. During the first 5 min the larvae were seen to be moving about actively, and only an occasional cell was seen attached to them. By 10 min, the PMN had formed larger clumps which were partly trapping the larvae and the larvae were beating about actively with their free ends, apparently trying to free themselves from the cells. By 20 min and later, most larvae were completely encased and immobilised by the cells. At times, five or six larvae were enclosed by one cell clump. Very few larvae were completely unaffected by the process of entrapment.

In the tissue the interaction between invading organism and the PMN probably occurs only in a restricted fashion. But, since neutrophils are the most numerous scavengers of the body and since they do not have to be sensitised to function, they must be considered the first line of defence against parasites. Within a brief time, they then secrete a substance that attracts eosinophils to the site. Compared to neutrophils, these cells contain larger amounts of certain enzymes (histaminase¹⁹, arylsulphatase²⁰, and myeloperoxidase²¹) and of basic proteins²² which may enable them to modulate the local inflammatory event in a way more favourable to the host.

It is important to remember that the release of ECF from the PMN is a very specific process with regard to the stimulus—phagocytosis of certain bacteria, has no such effect (data not shown). In addition, inhibitory factors in cells¹⁷ and tissues²³ may modulate the influx of eosinophils to inflammatory sites, and the final outcome of the neutrophil-associated inflammatory process may depend on the complex interaction of several factors.

In the light of these results, the induction of eosinophilia during parasitic infestations may be viewed as follows. First, PMN accumulate close to the parasite and, during the process of phagocytosis, release ECF which selectively attracts eosinophils and also some neutrophils. Local activation of complement and production of the neutrophil and eosinophil chemotactic fragment C5a (ref. 7), will also play a part early on in sustaining the influx of both cell types. This latter line of defence may be continuously active during the subsequent course, especially when circulating immune complexes²⁴ become available as an additional means of complement activation. Finally, lymphocyte- and mast cell-dependent mechanisms of induction of eosinotaxis are activated after sensitisation and production of specific IgE has occurred^{25,26}. It may well be that the response produced at this time is more effective in evoking and sustaining eosinophilia and in eliminating the invading organism. But the mechanisms induced at earlier times may serve to abort very mild infestations and/or to set the stage for a better immune response and a favourable outcome for the host.

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Novel type of murine B-cell lymphoma

ALTHOUGH several murine T-cell lymphomas have been well characterised (reviewed in ref. 1), only a single group of murine B-cell lymphomas, the Abelson lymphomas, has been described². The Abelson lymphoma arose in a steroid-treated BALB/cCr mouse inoculated with Moloney leukaemia virus², expresses surface immunoglobulin^{3–4} and can be transmitted by an infectious murine leukaemia virus². We report here a new IgM-bearing B-cell lymphoma distinct from the Abelson lymphoma in its induction, pathogenesis and antigenic characteristics and which has many features in common with the widely studied guinea pig L₂C lymphoma^{5–6}.

The phenomenon reported here was observed during the selection of a new strain of mice—B10-H-2^a H-4^bp/Wts⁷. This 'double congenic' strain differs from the background strain C57BL/10 (H-2^b, H-4^a) at two segments of its genome; one surrounding the H-2 locus and another surrounding the H-4 locus. When B10-H-2^a H-4^bp/Wts mice were immunised with sheep erythrocytes and their spleen cells or sera were transferred to syngeneic or F₁ hosts (protocol previously described⁸), a lymphomatous disease resulted. Symptoms suggesting a lymphomatous disease occurred in more than 50% of the cell and serum recipients within 150 d of transfer. Strains congenic to B10-H-2^a H-4^bp/Wts at the H-4 (B10.A; H-2^a H-4^a) and H-2 (B10.129(21M); H-2^b, H-4^b) loci did not develop lymphoma when treated in the same manner.

The disease is characterised by hypersplenomegaly, lymph node enlargement and whitish nodes in the liver. The primary disease process does not seem to involve the thymus or bone marrow and the serum from tumour-bearing animals does not seem abnormal on electrophoresis. Several of these primary tumour-bearing animals have been observed and one B10-H-2^a H-4^bp/Wts tumour was selected for transplantation and characterisation.

The cell surface antigens expressed by the lymphoma cells are presented in Tables 1 and 2. When these transplanted ascites tumour cells were assayed for surface immunoglobulin with a polyvalent rabbit anti-mouse immunoglobulin, more than 80% of them were consistently found to be positive for Ig by indirect immunofluorescence (Table 1). Studies with heavy-chain-specific reagents demonstrated that most if not all of these Ig+ cells specifically expressed IgM, and not IgA or IgG. Qualitatively, the large lymphoblast-like tumour cells demonstrated very bright diffuse fluorescence indicating a high

surface concentration of IgM. Attempts to kill the lymphoma cells with rabbit anti-mouse immunoglobulin and complement were unsuccessful; yet, this antiserum induces strong agglutination of tumour cells (unpublished observation).

That this murine lymphoma does not bear thymus cell antigens is conclusively demonstrated by the results presented in Table 2. Although the rabbit anti-mouse brain antiserum used to detect mouse T cells reacts with most of the tumour cells by indirect immunofluorescence, this phenomenon does not depend on a T-cell-specific antigen. Absorption of the rabbit anti-mouse brain antiserum with tumour cells does not remove the antibodies directed at normal mouse thymocytes. Additionally, absorption of the rabbit anti-mouse brain with thymocytes does not remove its reactivity with tumour cells; yet, does remove the anti-T-cell antibodies. The nature of this cross-reactive antigen is being investigated. Negative results from dye exclusion cytotoxicity assays with a specific anti-Thy 1.2 antiserum, as well as the rabbit anti-mouse brain, further substantiate that this tumour is not of thymic origin (Table 2).

Our preliminary evidence clearly distinguishes the B10-H-2^a H-4^b/Wts B lymphoma from the only other characterised group of murine B-cell lymphomas, the Abelson lymphomas. There are several important differences. First, induction: the lymphoma reported here is induced in recipients of sera or cells from immunologically stimulated B10-H-2^a H-4^b/Wts donors. Cell transfers from other congenic strains do not induce tumorigenesis. Conversely, Abelson lymphoma was induced in a BALB/cCr mouse treated with an immunosuppressant and inoculated with Moloney leukaemia virus². Second, pathogenesis: the B10-H-2^a H-4^b/Wts lymphoma mainly involves the peripheral lymphoid organs, in particular the spleen. This contrasts with the Abelson lymphoma which primarily involves the bone marrow cells and the meninges¹¹. Third, antigenic characteristics: although both Abelson B-cell lymphoma and the lymphoma reported here bear surface immunoglobulin, at least their quantity of expression differs. Sklar *et al.*³ have reported that cells infected with Abelson virus express surface immunoglobulin; yet, surface immunoglobulin can only be detected by very sensitive detection methods⁴ and only on a minor percentage of these tumour cells³. This is not the case with the B10-H-2^a H-4^b/Wts lymphoma. Ninety per cent of these cells show very bright fluorescence with an anti- μ specific antiserum (Table 1). These lymphoid cells are probably monoclonal, and the nature of the idiotypic determinants of this surface IgM is being investigated.

Table 1 Expression of surface immunoglobulin by B10-H-2^a H-4^b/Wts lymphoma cells

Primary antisera	% Immunofluorescence		
	Spleen	Thymus	Tumour
Rabbit anti-mouse Ig	35	<1	83
Goat anti- μ *	45	<1	90
Goat anti- α *	<1	<1	<1
Goat anti- γ *	10	<1	<1

Indirect immunofluorescence was performed as follows. 5×10^6 cells were suspended in 0.1 ml of the appropriate primary antiserum and incubated for 30 min at 4 °C. Cells were washed extensively in cold phosphate-buffered saline (PBS) containing 0.1 M Na₂S₂O₃. Cells were then resuspended in 0.1 ml of the appropriate fluorescein-conjugated secondary antiserum and incubated for 30 min at 4 °C. All cells were washed extensively in cold PBS-0.1 M Na₂S₂O₃, resuspended in a small volume of PBS-0.1 M Na₂S₂O₃ containing 50% glycerol, and observed under a Zeiss microscope equipped for fluorescent microscopy. Results are expressed as percentage of cells positive for fluorescence/total cells counted. Controls for phagocytosis and adherence of Ig to Fc receptors consisted of spleen, thymus and tumour cells incubated in the second antiserum only. Less than 1% of the control cells were positive. All antisera were diluted in PBS-0.1 M Na₂S₂O₃ to prevent capping. Rabbit anti-mouse Ig was prepared by standard methods and was used at a 1:30 dilution. The fluorescein-conjugated goat anti-rabbit Ig (Meloy, Springfield, Virginia) was diluted 1:40.

*Heavy-chain-specific antisera prepared in goats (Meloy) were diluted 1:20 in PBS-0.1 M Na₂S₂O₃. The second antisera, fluorescein-conjugated rabbit anti-goat Ig (Meloy) were diluted 1:40 before use.

Table 2 Failure to detect T-lymphocyte antigens on the B10-H-2^a H-4^b/Wts murine lymphoma

Antisera	% Immunofluorescence			% Cytotoxicity		
	Spleen	Thymus	Tumour	Spleen	Thymus	Tumour
anti-Thy. 1.2	ND	ND	ND	30	100	<1
Rabbit anti-mouse brain*	34	100	90	35	100	<1
Rabbit anti-mouse brain absorbed with thymocytes†	ND	<1	88	<5	<1	ND
Rabbit anti-mouse brain absorbed with tumour cells‡	ND	100	<10	15	90	ND

Indirect immunofluorescence was performed as described in Table 1. Controls for phagocytosis and adherence of Ig to Fc receptors showed that less than 1% of the cells were positive. Dye exclusion cytotoxicity was performed using a minor modification of the technique described by Frelinger *et al.*⁹. Guinea pig complement (Gibco) was the source of complement at a dilution of 1:8. Controls, consisting of cells with antiserum or complement alone, were consistently negative. NIH Contract anti-Thy 1.2 (AKR anti-C3H) antiserum was provided by the Research Resources Branch of NIH. This antiserum killed 100% of the control normal thymocytes at a dilution of 1:10. ND, not determined.

*Rabbit anti-mouse brain antiserum was produced in our laboratory by the technique of Golub¹⁰. For indirect immunofluorescence it was used at a dilution of 1:20 and for cytotoxicity at a dilution of 1:10.

†Rabbit anti-mouse brain antiserum (0.6 ml, 1:10 dilution) was absorbed twice with three normal thymus equivalents for 30 min at 25 °C and used at a dilution of 1:10.

‡Rabbit anti-mouse brain antiserum (0.6 ml, 1:10 dilution) was absorbed twice with 0.2 ml of packed tumour cells for 30 min at 25 °C and used at a dilution of 1:10.

The B10-H-2^a H-4^b/Wts lymphoma is interesting from another viewpoint. Because only cells or sera from immunologically stimulated B10-H-2^a H-4^b/Wts mice seem to be able to induce this disease, something involving the combination of the H-2^a and H-4^b chromosomal segments in association with an immune stimulus may make this phenomenon possible. Recently a second B-cell lymphoma, similarly induced in B10-H-2^a H-4^b/Wts mice, has been established in transplantation and found to bear surface IgM with an idotype distinct from that of the first tumour. Work is in progress to characterise these tumours and to define the genetic, virological and immunological aspects of these B-cell lymphomas. To add to the well known L₂C guinea pig leukaemia, these murine B-cell tumours provide another excellent model for a similar human malignancy, chronic lymphocytic leukaemia. The murine model has advantages over the guinea pig system in that large experimental groups can be used conveniently, comparisons can be made between different individual tumours and the genetics of the mouse have been much better defined.

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Induction of antigen-specific human suppressor T lymphocytes *in vitro*

INTENSIVE studies in a variety of animal species have led to the recognition that cell interaction is important for the generation of an immune response¹. The participation of macrophages and of T and B lymphocytes in the process of antibody formation has been demonstrated both *in vivo* and *in vitro*^{2,3}. More recently, attention has been focused on the mechanism(s) regulating the development and expression of immune reactivity, leading to the discovery in a number of animal species of subpopulations of T cells with different functions, such as helper cells (Th) and suppressor cells (Ts) (refs 4-6). Dosch and Gelfand⁷ recently reported that the induction of plaque forming cells (PFC) in human tonsillar or blood lymphocytes is abolished if a (relatively) high antigen dose is used and we have confirmed this finding using the antigens sheep red blood cells (SRBC), ovalbumin (OA) and haemocyanin (Hc)⁸. We have extended these studies and report here the *in vitro* generation of antigen-specific Ts cells in man.

Human peripheral blood lymphocytes (PBL), isolated by density centrifugation⁹ were cultured in tubes for 6 d with or without antigen (SRBC or OA). The cells were then collected, washed and transferred to monolayers of untreated SRBC or of OA-coated SRBC. Primed lymphocytes secreting specific antibody can be recognised by plaque formation. The specificity of this *in vitro* immune response has been firmly documented^{7,8}. The magnitude of the PFC response is antigen dose-dependent, as illustrated in Table 1. Optimal results in the conditions used were obtained at an antigen concentration of $3 \mu\text{g ml}^{-1}$ for OA and at a lymphocyte:SRBC ratio of 1:1. The low number of PFC found in cultures stimulated with a high dose of antigen suggested the generation of suppressor cell activity. However, in these conditions B cells might be inactivated by the high antigen concentration without interference of Ts. To differentiate between these two possibilities PBL were cultured for 24 h with a high dose of OA ($100 \mu\text{g ml}^{-1}$), the cells were collected, washed and added to an equal number of autologous PBL. The cell mixture was cultured in standard conditions (see Table 1) at optimal antigen concentration ($OA 3 \mu\text{g ml}^{-1}$) for 6 d and the number of PFC determined. The degree of suppression in these cultures ranged from 62 to 94%, with an average of $80.7 \pm 14.5\%$ (mean $\pm$ s.e.m.) in five different experiments (Table 2). These findings indicate that the low number of PFC generated in cultures

Table 2 Effect of *in vitro* induced suppressor cells on the anti-OA PFC response

Expt no.	Day 1 PBL*	Day 2 Suppressor cells†	Day 6 PFC per 10^6 lymphocytes	% Suppression‡
1	10×10^6	—	1,810	
	5×10^6	5×10^6	245	86.5
2	10×10^6	—	737	
	5×10^6	5×10^6	233	68.3
3	10×10^6	—	857	
	5×10^6	5×10^6	48	94.3
4	10×10^6	—	1,236	
	5×10^6	5×10^6	91	92.6
5	10×10^6	—	5,357	
	5×10^6	5×10^6	2,020	62.3

*In control experiments 10^7 PBL were treated as outlined below except that the antigen dose used was $OA 3 \mu\text{g ml}^{-1}$.

†In order to generate suppressor cells 10^7 lymphocytes were cultured in the presence of $OA 100 \mu\text{g ml}^{-1}$ in 10 ml RPMI-1640 containing antibiotics L-glutamine and 10% AB serum for 24 h. Cells were collected, washed twice in RPMI-1640 and 5×10^6 of these cells were added to 5×10^6 PBL that had been primed with $OA 3 \mu\text{g ml}^{-1}$ 24 h earlier and cultured for an additional period of 5 d.

‡The % suppression was calculated according to the formula:

$$100 - \frac{\text{No. PFC per } 10^6 \text{ lymphocytes in suppressed cultures}}{\text{No. PFC per } 10^6 \text{ lymphocytes in normal cultures}} \times 100$$

with high doses of OA is the result of a suppressive action of PBL induced by contact of these cells with OA in high concentrations. To identify the cell type responsible for the suppression of *in vitro* antibody response, PBL were depleted of adherent cells and separated into T and non-T cells (see legend to Table 3). The isolated T lymphocytes and the non-T cells (B and null cells) were supplemented with 5% adherent cells and incubated for 24 h with $OA 100 \mu\text{g ml}^{-1}$. The cells of these two different populations were then washed and added to normal PBL primed with $OA 3 \mu\text{g ml}^{-1}$ 24 h

Table 3 The nature of the *in vitro* induced suppressor cells

Cell mixtures added to PBL with antigen	Treatment	PFC response to OA (PFC per 10^6 lymphocytes)		
		Expt 1	Expt 2	Expt 3
T cells	—	906	686	616
Non-T cells*	—	—	—	—
T cells	$100 \mu\text{g OA}$	267(70.5)†	240(65)	185(70)
Non-T cells	—	—	—	—
T cells	—	715(21.1)	750(—9.3)	608(1.3)
Non-T cells	$100 \mu\text{g OA}$	—	—	—

PBL, isolated by density gradient centrifugation, were depleted of non-lymphoid mononuclear cells by incubation in Falcon flasks (45 min at 37°C) in RPMI-1640 supplemented with 20% foetal bovine serum (PBL_{acc}). The adherent cells (monocytes, macrophages) were recovered by gently scraping with a rubber policeman, washed twice in Ca^{2+} - and Mg^{2+} -free Earle's balanced salt solution supplemented with EDTA (0.02 mM) and resuspended in culture medium. The non-adherent cells were separated into T cells and non-T cells according to the method of Moretta *et al.*¹⁰. The isolated T cells and the non-T cells (B and null cells) were supplemented with 5% adherent cells and incubated for 24 h with $OA 100 \mu\text{g ml}^{-1}$ or without antigen in culture medium (see Table 1). The cells were then washed twice with RPMI-1640. Since it has been found that the concentration of OA necessary for the induction of maximal numbers of PFC increases with the numbers of T cells present in a given cell suspension^{7,8} T cells and non-T cells, previously incubated with $OA 100 \mu\text{g ml}^{-1}$ were reconstituted with normal non-T cells and T cells respectively to obtain cell mixtures with T:B ratios equally to those of normal PBL suspension. 5×10^6 Lymphocytes of these cell mixtures were added to 5×10^6 PBL_{acc} reconstituted with 5% macrophages and incubated with $OA 3 \mu\text{g ml}^{-1}$ 24 h earlier. After 5 d PFC assay was performed⁷.

*T-cell contamination in the non-T cell population was 3.5%, < 0.5% and 1.5% respectively in Expts 1, 2 and 3.

†The values in parentheses represent the percentages of suppression calculated according to the formula in the legend to Table 2.

Table 1 Role of antigen concentrations in the PFC response of PBL

OA ($\mu\text{g ml}^{-1}$)	PFC per 10^6 lymphocytes	Lymphocyte:SRBC ratio	PFC per 10^6 lymphocytes
0.3	700(150–1,600)	10:1	299(1,805–560)
1.0	1317(920–2,000)	1:1	1538(1,026–2,100)
3	1418(600–3,000)		
30	230(25–800)	1:10	240(37–620)

For the induction of PFC to OA and SRBC, cultures were set up in 17×100 mm tissue culture tubes (Falcon) containing 5×10^6 PBL in 10 ml RPMI-1640 medium supplemented with 10% heat inactivated AB serum (of a pooled stock of three donors), penicillin (100 IU ml^{-1}), streptomycin ($100 \mu\text{g}$), Fungizone ($25 \mu\text{g ml}^{-1}$), L-glutamine (2 mM) and antigen at different concentrations. When SRBC were used as antigen, the AB serum was absorbed three times with SRBC to prevent pseudo-plaque formation¹⁰. The cultures were kept in a humidified atmosphere of 5% CO_2 in air. After culture for 6 d cells were collected, washed twice in RPMI-1640 at 4°C and assayed for PFC according to the method of Dosch and Gelfand⁷. The data shown are expressed as the arithmetical mean of values obtained in 10 individual experiments. Within each experiment determinations at the various antigen doses were done in six fold. Values in parentheses represent the ranges.

earlier (the suppressive effect in these conditions is optimal, data not shown). After 5 d of culture PFC assay was performed. The results (Table 3) show clearly that after incubation for 24 h with a high dose of OA, non-T cells are not able to suppress PFC response *in vitro*. In contrast, suppressive activity can be induced in identical conditions in a purified T-lymphocyte population. This might be interpreted as the result of activation or generation of Ts. It was realised that a high concentration of antigen, carried over by cells previously incubated with the antigen, might have induced B-cell unresponsiveness. There are, however, several arguments that counter this possibility. First, in contrast to T cells, non-T cells after incubation with OA $100 \mu\text{g ml}^{-1}$ failed to suppress the anti-OA PFC response. Therefore, neither B lymphocytes nor adherent cells (macrophages) are able to carry over sufficient amounts of antigen to inhibit PFC formation. Antigen carry-over by T cells is therefore highly unlikely. Second, Cobi Heynen *et al.* (in preparation) found evidence that a major subpopulation of T cells bearing a receptor for the Fc fragment of IgM ($T\mu$) is not able to suppress an anti-OA PFC response after treatment with high concentrations of this antigen. Taken together these results fail to support induction of B-cell unresponsiveness by the carry-over of high antigen concentrations as the predominant mechanism of the observed suppression.

Table 4 Specificity of suppression by PBL treated with OA

PBL* treated with ($100 \mu\text{g ml}^{-1}$)	Antigen in culture	PFC response against	% Suppression of PFC response†	Expt 1	Expt 2	Expt 3
OA	SRBC	SRBC	—8	26.7	10.8	
OA	OA	OA	92	92.6	73.9	

*Suppressor cells were generated as described in the legend to Table 2.

†Percentage suppression was calculated according to the formula in Table 2 legend.

The suppression obtained in our system is antigen specific, as Ts induced with OA did not suppress PFC formation against SRBC (Table 4). Also, Ts induced by incubation with high numbers of SRBC specifically suppress PFC response against SRBC but have no effect on OA-specific PFC (data not shown). It should be emphasised that the suppressive effect described here is on the IgM response. Since it has been found in mice that IgM responses are more difficult to suppress than secondary IgG formation^{11,12}, it would be interesting to study the effect of induced Ts on restimulated cultures. Attempts are currently being made to obtain an anamnestic immune response *in vitro*.

The possibility of inducing *in vitro* antigen specific suppressor cells might have far reaching implications in immune manipulation in man.

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Glutamate neurotoxicity and Huntington's chorea

McGEER and McGeer¹ recently reported that an intrastriatal injection of glutamate results in biochemical changes in brain similar to those associated with Huntington's chorea. They postulated that glutamate, a putative excitatory transmitter and an 'excitotoxin' abundantly present in brain may have a role in the pathophysiology of Huntington's chorea. Their evidence for this postulate must be considered very preliminary, however, as the biochemical changes induced by a 50 nmol dose of glutamate were not striking and it was not determined whether these changes were accompanied by neuronal degeneration¹. We have injected various doses of glutamate directly into the rat striatum and examined the striatum for histopathological changes 21 d later. Here we report that doses of glutamate much higher than 50 nmol definitely cause striatal neurones to degenerate, whereas no neurotoxic reaction results from injecting equally high doses of control compounds such as NaCl or γ -aminobutyric acid (GABA).

Glutamate and its several neuroexcitatory analogues, when administered systemically to experimental animals, destroy neurones in certain brain regions which lack adequate blood brain barriers to these compounds². Certain analogues, such as kainic acid (KA), *N*-methyl aspartic acid (NMA) and homocysteic acid (HCA) are more powerful than glutamate in either excitatory or toxic activity^{2,3}. The introduction of tiny amounts of these potent analogues by microinjection directly into the rat brain destroys local neurones without damaging axons passing through or terminating in the region⁴; lesion size is directly proportional to the known excitatory potency of the injected compound, the order of potencies being KA>NMA>HCA. A single injection of KA (5–10 nmol) into rat striatum results in rapid loss of striatal neurones^{5–7} and specific changes in transmitter enzymes^{1,5} similar to those known to occur in Huntington's chorea. This suggests that the more potent analogues of glutamate may be useful tools for studying Huntington's chorea. The findings reported here are consistent with the related but potentially more important proposal¹ that glutamate itself might have a role in the loss of striatal neurones in Huntington's chorea.

Twenty-four adult male Wistar rats (Harlan) weighing 250 g were used in these experiments. Monosodium L-glutamate (Glu) and GABA were obtained from Sigma. All compounds were introduced stereotactically into the striatum as previously described⁷ through a 30 gauge cannula by the method of Swanson *et al.*⁸. Glutamate was dissolved in sterile H₂O in various concentrations to permit testing four doses (50, 150, 500 and 1,000 nmol) in a volume of 1 μl . Aqueous solutions of two control compounds, NaCl and GABA were administered at 1,000 nmol to match the highest dose of glutamate used. The glutamate and GABA solutions were at pH 7.0 and NaCl was at pH 6.1. At least four animals were used to test each dose of each compound. Each animal, under pentothal anaesthesia, was given a single 1- μl injection of solutions containing one of the three compounds. The solutions were injected slowly over a 5-min

period and the cannula was left in place for an additional 10 min before removal to prevent retrograde movement of the injected solution up the cannula tract. Animals were killed 21 d after injection by glutaraldehyde-paraformaldehyde perfusion fixation under chloralhydrate anaesthesia and 3/4-mm serial slabs collectively encompassing the entire striatum were additionally fixed in osmium tetroxide and processed for combined light and electron microscopic examination by methods previously described^{9,10}.

None of the striata injected with GABA or NaCl had detectable histopathological changes other than minor changes expected from cannula insertion. The injection site could be identified by a small scar, but this was surrounded on all sides by healthy looking neurones in normal numbers. The appearance of a GABA injection site is illustrated in Fig. 1. The failure of NaCl injection to induce a neurodegenerative reaction in rat brain is reported elsewhere²².

In the striata of animals which received 50 or 150 nmol of glutamate there were indications about the injection site of a more extreme tissue reaction than was induced by GABA or NaCl, but definitive evidence for neuronal loss was lacking. It should be noted, of course, that the deletion of a very small number of neurones would be difficult to demonstrate with any confidence. All animals which received 500 nmol of glutamate had a decidedly larger area of tissue disturbance which we judged to be evidence for a neurotoxic reaction, based on the abundant reactive cells present and of the reduced number of neuronal profiles detectable. We estimated that approximately 5–10% of the total striatal population of neurones was deleted by 500 nmol of glutamate. A much larger area of tissue disturbance was evident in all animals which had received an injection of 1,000 nmol of glutamate. Approximately 30% of the total striatal neuronal population was deleted by this dose of glutamate. There were numerous reactive cells distributed about the lesioned area and neuronal profiles were absent but axonal bundles remained intact (Fig. 2). Thus, the effect seemed to be the characteristic dendrosomatotoxic but axon-sparing type of neurodegenerative effect which excitotoxic compounds are known to produce^{4,5,7,10,11}.

Our observation that an intrastriatal injection of glutamate destroys striatal neurones is consistent with the finding of McGeer and McGeer¹ that injecting glutamate into the

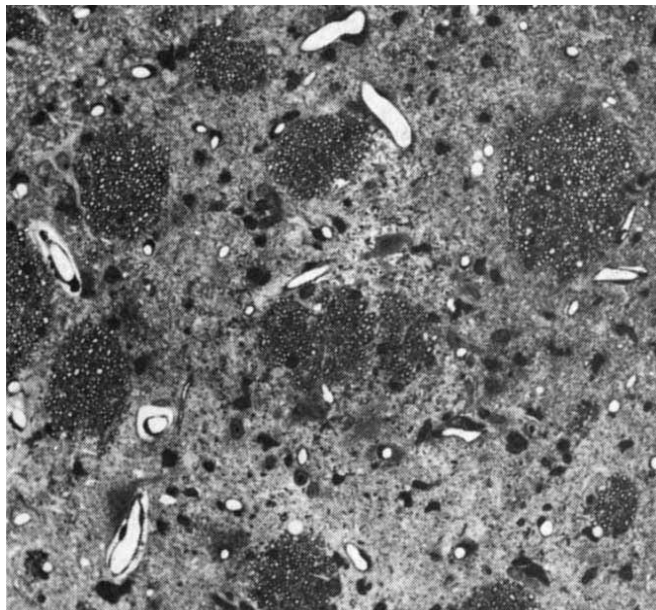


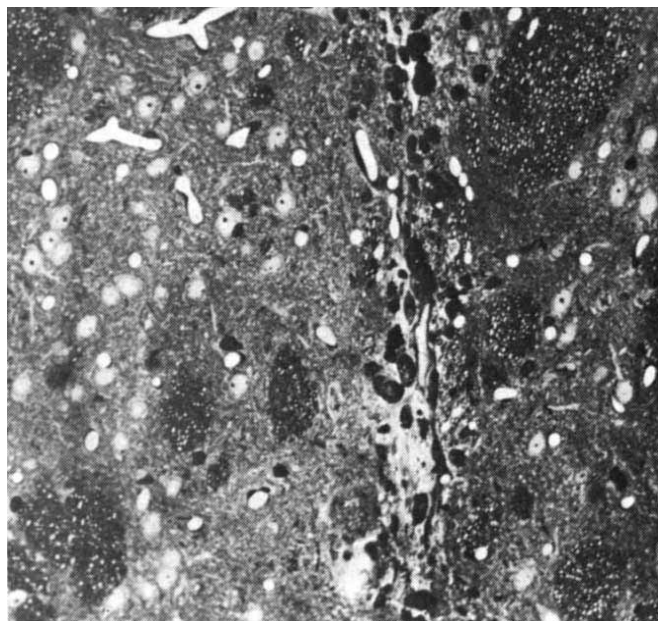
Fig. 2 A light micrograph depicting a region of striatum about 500 μ m away from the site where 1,000 nmoles of Glu was injected 21 d previously. Although all intrinsic striatal neurones have degenerated beyond recognition, bundles of axons passing through the striatum remain intact and have a normal appearance ($\times 280$).

striatum causes a loss of the transmitter-synthesising enzymes, cholineacetyltransferase and glutamic decarboxylase which are considered markers for striatal neurones. It is puzzling, however, that we detected significant neuronal loss from doses of glutamate substantially higher than the 50 nmol which produced enzyme changes in the experiments of McGeer and McGeer. Presumably, a reduction in transmitter marker enzymes would occur only to the extent that neurones containing these enzymes are destroyed. Additional studies are needed to clarify whether such an expectation is warranted and to explain the apparent discrepancy between our findings and those of McGeer and McGeer¹.

Although our results suggest that glutamate is a weak neurotoxin compared to KA, we suspect this may be due in part to mechanisms in normal brain which function to protect neurones more effectively from glutamate than from KA. For example, the high affinity uptake system which, by removal of glutamate from excitatory receptor loci may control its excitotoxic potential, is relatively inaccessible to KA¹². This inactivation system may be efficient enough in normal striatum to prevent glutamate, unless injected in large amounts, from destroying local neurones. If, in the course of an individual's life, this vital homeostatic mechanism became dysfunctional, however, glutamate released at excitatory synapses might gradually accumulate in the synaptic cleft region in sufficient concentration to cause excitotoxic degeneration of glutamergically innervated neurones. In view of accumulating evidence^{13–17} suggesting that striatal neurones receive massive input from terminals utilising glutamate as transmitter, it is reasonable to propose that glutamate may be involved in the degeneration of striatal neurones in Huntington's chorea. Indeed, for heuristic purposes, we would advance the more specific proposal that an adult-onset disturbance in the transport system for intracellular reuptake of glutamate might underlie this neurodegenerative syndrome. This may be a testable hypothesis, depending on the technical feasibility of assaying the high affinity glutamate uptake capacity of striatal tissue obtained at autopsy from brains of patients with Huntington's chorea.

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Fig. 1 A light micrograph depicting a region of striatum where 1000 nmol of GABA was injected 21 d previously. A scar is detectable where the cannula tip penetrated the striatal tissue but healthy neurones in normal numbers surround the scar ($\times 280$).



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Morphine and β -endorphin inhibit release of noradrenaline from cerebral cortex but not of dopamine from rat striatum

β -ENDORPHIN is a large polypeptide containing 31 amino acid residues, the first five of which are identical with methionine-enkephalin¹ which has potent naloxone-sensitive analgesic properties² and opiate activity in receptor binding assays in guinea pig ileum^{3,4}. It has been shown that morphine depresses the potassium-induced release of ³H-noradrenaline in the rat cerebral cortex⁵ and of ³H-dopamine in the rat striatum⁶. These effects are blocked by the opiate antagonist naloxone and are probably due to the activation of presynaptic inhibitory opiate receptors located in noradrenergic and dopaminergic nerve endings of the rat brain. While met-enkephalin reproduced the effects of morphine in rat cortex slices⁷, it failed to reduce the release of dopamine from the rat striatum⁶. In view of these results, it was considered of interest to examine under similar experimental conditions, the effects of morphine and of β -endorphin on the potassium-stimulated release of ³H-dopamine from striatal slices and of ³H-noradrenaline from cerebral cortex slices of the rat⁸. In contrast to Loh *et al.*⁶, who more recently have not been able to reproduce their data (E. L. Way, personal communication), we find no evidence for the inhibition of the potassium-stimulated overflow of ³H-dopamine by β -endorphin and morphine in the striatal slices of the rat.

Male rats of 150 to 200 g were killed by decapitation. Slices of 0.3 mm thickness were obtained from the rat corpus striatum and occipital cerebral cortex with a McIlwain tissue chopper. The slices were transferred to the incubation medium in an open lucite cylinder with a nylon mesh fitted to the bottom as a small basket⁸. The whole system was placed in a 30-ml beaker with 5 ml of Tyrode solution containing 61 μ M ascorbic acid and 69 μ M EDTA equilibrated with a 5% CO₂ in O₂ and maintained at 37 °C.

Striatal and occipital cortex slices were incubated for 30 min with 2 μ M ³H-dopamine and 0.9 μ M ($\pm$) ³H-noradrenaline, respectively. The tissue was washed by transferring to successive beakers, and the spontaneous outflow of radioactivity into the medium was followed for 65 min before the release of the labelled transmitter was elicited by 1 min exposure to 20 mM K⁺. Two periods of stimulation with K⁺ were applied in each experiment, S₁ and S₂. The interval between S₁ and S₂ was 40 min. The overflow of the labelled transmitter elicited by

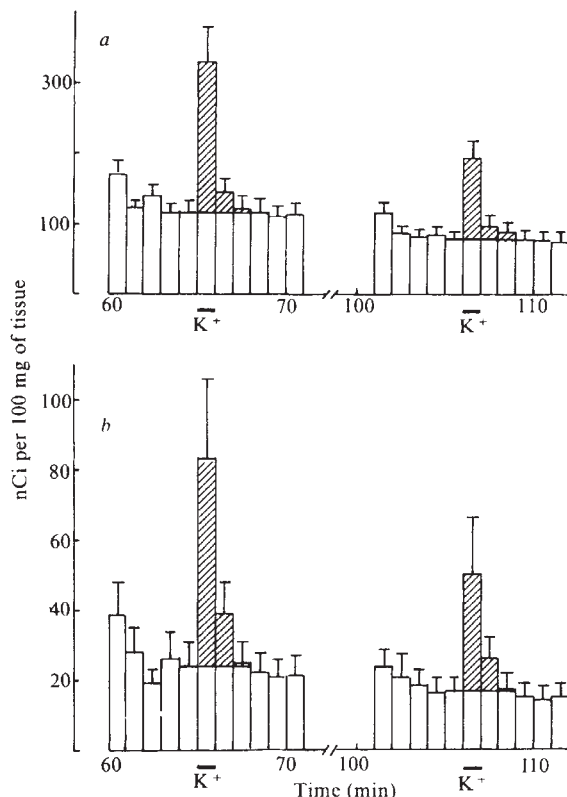


Fig. 1 Release of radioactivity from slices of rat corpus striatum and occipital cortex during exposure to 20 mM K⁺. Ordinate, fraction of the total radioactivity in the tissue released per min (nCi per 100 mg of tissue), abscissae, time (in min) after the end of the incubation with either ³H-noradrenaline (NA) or ³H-dopamine (DA). The open columns represent the spontaneous outflow of total radioactivity in 1-min samples. The horizontal bar indicates the 1-min exposure to 20 mM K⁺ which was repeated 40 min later. The shaded areas indicate the increase in release of radioactivity induced by K⁺. *a*, release of ³H-DA from slices of rat corpus striatum; values are the mean $\pm$ s.e.m. ($n = 13$); *b*, release of ³H-NA from slices of rat occipital cortex, values shown are the mean $\pm$ s.e.m. ($n = 7$). Tissue tritium content at the end of the experiment was $3,546 \pm 616$ nCi per 100 mg ($n = 13$) in the striatum and 938 ± 98 nCi per 100 mg ($n = 7$) in the occipital cortex; n = number of experiments per group.

potassium was expressed as per cent of the tissue stores: total nCi released as a fraction of the total radioactivity present in the tissue at the onset of stimulation⁹. Figure 1 shows the pattern of ³H-transmitter release elicited by each of the two consecutive periods of exposure to potassium in the rat striatum (Fig. 1*a*) and in rat occipital cortex slices (Fig. 1*b*).

Table 1 shows that in contrast with the cerebral cortex slices,

Table 1 Influence of the calcium concentration in the medium on the potassium-induced release of ³H-transmitter from slices of rat occipital cortex or corpus striatum

Tissue	<i>n</i>	Per cent tissue stores*		S ₁ as % of S ₂
		S ₁ Ca ²⁺ = 1.78 mM	S ₂ Ca ²⁺ = 1.78 mM	
Occipital cortex	7	3.88 $\pm$ 0.74	4.63 $\pm$ 1.27	96.43 $\pm$ 9.19
Striatum	13	4.31 $\pm$ 0.68	5.14 $\pm$ 0.86	100.54 $\pm$ 18.18
Ca ²⁺ = 0 mM				
Occipital cortex	4	0.19 $\pm$ 0.06	4.06 $\pm$ 1.45	5.27 $\pm$ 1.08†
Striatum	8	1.97 $\pm$ 0.32	7.97 $\pm$ 2.09	38.01 $\pm$ 12.70‡

*Per cent of the total tissue radioactivity released by exposure to 20 mM K⁺.

S₁ corresponds to the first 1-min exposure to K⁺ and S₂ to the second, obtained 40 min after S₁.

Upper part, Tyrode solution with normal calcium; lower part, calcium-free medium during S₁.

Shown are mean values $\pm$ s.e.m.; n = no. of experiments.

† $P < 0.001$ ‡ $P < 0.02$ when compared against the corresponding controls (upper part of the table).

the release of the labelled transmitter elicited by K^+ from the rat striatum was not entirely calcium-dependent. In the absence of calcium, the release of 3H -noradrenaline induced by potassium from the cerebral cortex was practically abolished (Table 1). In the striatum, however, as much as 40% of the release elicited by K^+ was found to be calcium-independent. Figure 2 shows that morphine and β -endorphin significantly decreased the K^+ -induced release of 3H -noradrenaline from the cerebral cortex. The effects of both opiate receptor agonists were reversed in the presence of naloxone (Fig. 2). In contrast with these results, neither morphine nor β -endorphin were able to inhibit the release of 3H -dopamine from rat striatal slices (Fig. 3) even when tested in concentrations higher than those found to be effective in the cerebral cortex (compare Figs 2 and 3).

Our results support the view that presynaptic inhibitory opiate receptors are present in noradrenergic nerve endings of the rat cerebral cortex. In addition, we found that β -endorphin stimulates these presynaptic opiate receptors and that it is at least 10 times more potent than morphine in reducing 3H -noradrenaline release. The failure of morphine and of β -endorphin to reduce the release of 3H -dopamine from the striatum represents a conflict of evidence with a previous report⁶. It should be noted, however, that in our experimental conditions, we stimulated release with a 1 min exposure to 20 mM K^+ , while in the previous publication, 53 mM K^+ during 20 min was used⁶. We consider our experimental conditions (short pulse of a moderate potassium concentration) to mimic nerve stimulation more closely than a prolonged exposure to a rather high concentration of K^+ .

During a 1 min exposure to 20 mM K^+ , approximately 5% of the total tissue radioactivity was released from the striatum (Table 1). In separate experiments in which the slices were exposed to 40 mM K^+ for 5 min, $36.27 \pm 2.84\%$ of the total tissue radioactivity was released from the striatum (mean $\pm$ s.e.m. of four experiments). We consider that such a massive release of 3H -dopamine is not an adequate model for studies on transmitter release. It should be noted that for similar studies in the peripheral nervous system the fraction of the total tissue radioactivity released by nerve stimulation does not exceed 5% of the tissue stores⁹⁻¹². Attention should be drawn to the fact that even for a short lasting exposure to as little as 20 mM K^+ , more than one-third of the labelled transmitter released from the striatum represented a calcium-independent release. Under similar experimental conditions, the release of 3H -noradrenaline from the rat cortical slices was entirely calcium-dependent.

Fig. 2 Effects of morphine and β -endorphin on the release of 3H -noradrenaline elicited by potassium in the rat occipital cortex. Ordinate, ratio of fractional release (S_2/S_1); S_1 corresponds to the first 1-min exposure to 20 mM K^+ and S_2 to the second obtained 40 min after S_1 . Morphine was added 25 min before S_2 ; β -endorphin 10 min before S_2 and naloxone 35 min before S_2 . Values shown are mean $\pm$ s.e.m. of 4-7 experiments per group.

* $P < 0.001$ when compared against the untreated group.

† $P < 0.001$ when compared against morphine 10 μM .

‡ $P < 0.005$ when compared against β -endorphin 0.6 μM .

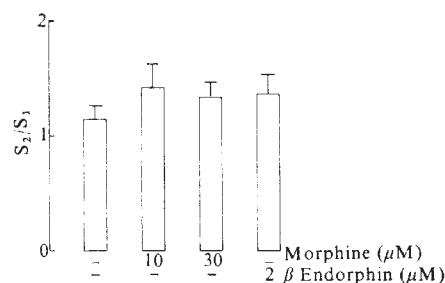
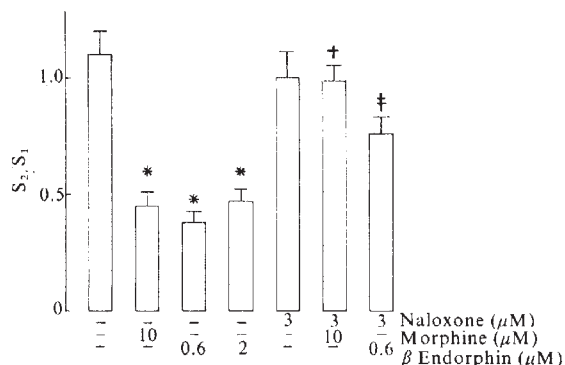


Fig. 3 Effects of morphine and β -endorphin on the release of 3H -dopamine elicited by potassium in rat striatal slices. Ordinate, ratio of fractional release (S_2/S_1); S_1 corresponds to the first 1-min exposure to 20 mM K^+ and S_2 to the second obtained 40 min after S_1 . Morphine was added to the medium 25 min before S_2 , and β -endorphin 10 min before S_2 . Values shown are mean $\pm$ s.e.m. of 6-11 experiments per group.

Our results regarding the 40% calcium-independent release of 3H -dopamine from the striatum are in disagreement with previous reports which demonstrated either total calcium dependence^{13,14} or only a 15% calcium-independent component¹⁵, although a 33% calcium-independent component for the potassium-induced release of 3H -dopamine from the rat striatum was reported by Dismukes and Mulder¹⁶. We have found that the extent of the calcium-independent component for 3H -dopamine release from the striatum (40%) was not affected by inhibition of monoamine oxidase *in vitro* or *in vivo* with pargyline. Under our experimental conditions 3H -dopamine release originated from reserpine-sensitive storage sites in the striatum. In support of this view it was found that pre-treatment with reserpine (5 mg kg^{-1} , 24 h before the experiment) reduced considerably the ability of the striatum to take up and store 3H -dopamine. In these experiments, exposure to K^+ was practically ineffective in eliciting 3H -dopamine release. It is concluded that β -endorphin and morphine reduce noradrenaline release from the rat cerebral cortex through the stimulation of presynaptic opiate receptors. Neither morphine nor β -endorphin reduced the release of dopamine from the rat striatum. In addition, a significant calcium-independent release of 3H -dopamine has been found in the rat striatum.

The magnitude of the calcium-independent component of 3H -dopamine release may vary with the strength and duration of the depolarising stimuli. Consequently, it is possible that the recent controversial reports on the effects of dopamine agonists and neuroleptics on dopamine release from the rat striatum may reflect experimental conditions with different degrees of calcium-independent release of 3H -dopamine^{13-14,16-18}. As a result of these conflicting reports, the presence of presynaptic dopaminergic receptors and their role in the regulation of dopamine release from nerve endings in the central nervous system remains an open question¹⁹.

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Quantal size is not altered by abrupt changes in nerve terminal volume

ACETYLCHOLINE (ACh) is released from stimulated nerve terminals in packets or quanta, each containing roughly 10,000 molecules^{1,2}, but we still do not know how the quanta are formed. A widely accepted hypothesis is that the ACh contained in vesicles found in the nerve terminal is released by exocytosis, while an alternative suggestion is the gated channel hypothesis. This takes into account the fact that the ACh which seems to be in free solution in millimolar concentrations in the terminals⁴ is probably the source of the ACh which leaks molecule by molecule from the resting terminals (only a small fraction of the spontaneous release is quantal^{5,6}). Other evidence against the vesicle hypothesis is that when nerves are exposed to radioactive precursors, the free ACh is labelled first, and with stimulation the newly synthesised ACh is released preferentially⁷. The free ACh could also be released in packets if stimulation opens gates on channels through which ACh diffuses from the terminals; with each opening of a channel, about 10,000 transmitter molecules might escape by moving out of the terminal down a concentration gradient. One way to test the gated channel hypothesis is to change abruptly the concentration of free ACh in the terminal, and so change the number of ACh molecules per quantum. This has been achieved in a molluscan, cholinergic neurone by injecting acetylcholinesterase into the cell body. The enzyme diffuses down to the terminals, hydrolyses the free ACh, and produces a transmission block⁸. Unfortunately, in this preparation it is not possible to determine the quantal size, so it is not certain that the block occurs because the quanta become smaller. Quantal size is readily estimated at the vertebrate neuromuscular junction, but it is not possible to inject substances into the fine nerve terminals and there does not seem to be a feasible way to

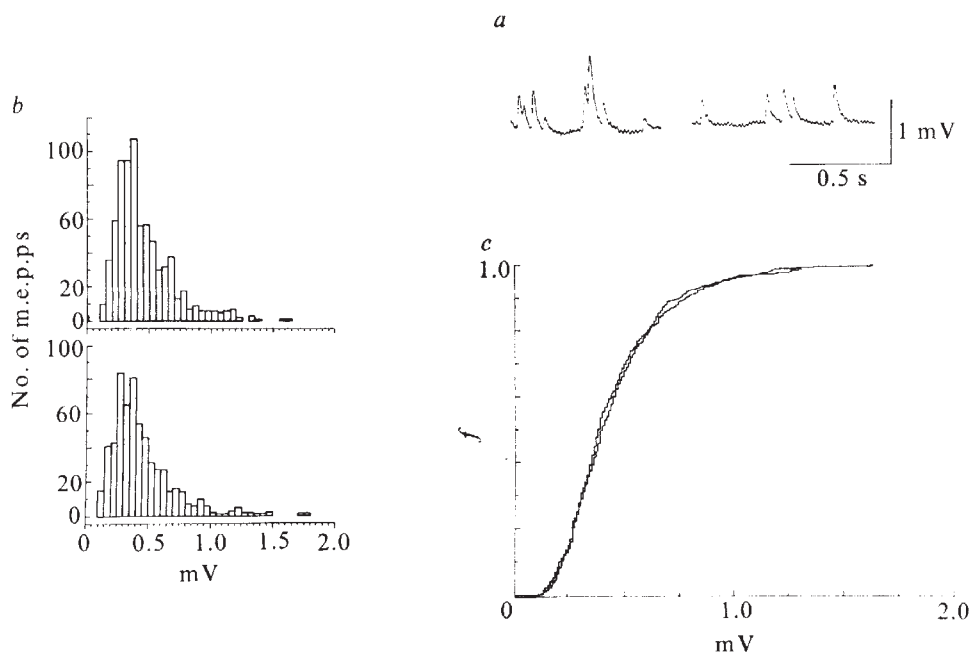
increase or decrease abruptly the amount of ACh in the terminal. An alternative approach, which I have used here, is to change the concentration of free ACh by moving water in or out of the nerve terminal, using osmotic pressure as the driving force^{9,10}.

To avoid problems with changing numbers of quanta released with each stimulus, spontaneously occurring miniature endplate potentials (m.e.p.ps) were used to estimate quantal size. The protocol for the first set of experiments was as follows; a sciatic nerve-sartorius muscle preparation from the frog *Rana pipiens* was immersed in Ringer solution made hypertonic by the addition of 250 or 500 mM sucrose. After a one hour equilibration a microelectrode was inserted at the endplate and m.e.p.ps were recorded. The microelectrode was then withdrawn until it was just outside of the fibre, the extracellular solution was aspirated from the bath, a fresh solution of normal tonicity was passed over the preparation, the micro-electrode was reinserted, and m.e.p.ps were recorded once again. The change in solution and repenetration took less than 20 s. The m.e.p.p. amplitudes were measured before and after the change in tonicity.

Successful experiments met a set of criteria; (1) the fibre was seen clearly and visual landmarks were good, so the probability was high that the microelectrode was reinserted into the same fibre; (2) the membrane potential did not deviate by more than 3 mV during the penetrations or with the repenetration, so the amplitudes did not have to be corrected for changes in membrane potential; (3) m.e.p.p. frequency was sufficiently low for some individual m.e.p.ps to be distinctly separated in time from their fellows, so that their amplitudes could be accurately measured. This criterion might seem to be impossible to meet. Hypertonic solutions are known to cause massive increases in m.e.p.p. frequency, an increase in tonicity of 100 to 150 mOsm often produces frequencies too high for the accurate counting of events; the records are far too jumbled for accurate amplitude measurements. It has been found, however, that with increases in tonicity above 450 mOsm l⁻¹ m.e.p.p. frequency begins to decline once again¹¹. Therefore, in Ringer containing 250 or 500 mM sucrose, some junctions can be found at which there are sufficient numbers of free-standing m.e.p.ps for accurate amplitude measurements.

From many attempts, seven experiments met the three criteria. An example is illustrated in Fig. 1. There is no significant difference between the mean amplitudes in hypertonic and in normal Ringer's solution. In both solutions, the amplitude

Fig. 1 *a*, Examples of m.e.p.ps recorded in Ringer (right hand trace) and in Ringer plus 500 mM sucrose (left hand trace). The Ringer contained (in mM): 110 NaCl, 2.0 KCl, 2.5 CaCl₂, 6 Na H₂PO₄ (hydroxymethyl) methyl-2-aminoethanesulphonic acid (TES) at pH 7.4 and 10⁻⁶ g ml⁻¹ neostigmine methylsulphate. Resting potential -78 mV. The m.e.p.ps were recorded on tape at 7½ inch s⁻¹ and played back at ½ inch s⁻¹ to a Hewlett Packard ink writer; *b*, amplitude histograms of the m.e.p.ps recorded in one experiment in Ringer (mean ± s.e.m. 0.45 ± 0.24, *n* = 755 (upper trace) and in Ringer plus 500 mM sucrose (0.47 ± 0.26, *n* = 665, lower trace); *c*, the cumulative distribution of amplitudes in the two solutions. The cumulative relative frequency, *F*, is plotted as a function of the amplitudes. The probability of the deviation between the two distributions occurring by chance is greater than 0.15.



distribution is skewed toward higher values and neither fits a normal probability distribution function. Therefore, the two distributions were compared further by plotting the cumulative probability distribution functions of the amplitudes and then comparing the two by the Kolmogorov-Smirnov statistic. The differences between the two are readily attributed to chance variation ($P > 0.1$). The records were carefully examined by eye to see if there was a transient change in amplitude immediately following the solution change, but none was detected. Furthermore, the regression line for the first 50 or 100 m.e.p.p. amplitudes following the solution change were calculated. The slopes of the lines were indistinguishable from zero (for 100 events in the experiment in Fig. 1, $b = 0.0001 \pm 0.00027$). Similar results were obtained in the six other successful experiments (in two of these the transition was in the opposite direction, from normal to hypertonic Ringer).

Before concluding that changes in terminal volume have no effect on quantal size, it is necessary to rule out the possibility that changes in tonicity have other, compensating effects on endplate physiology, so that real changes in the amounts of ACh released are not detected in the experiments. An increase in tonicity might decrease the sensitivity of the endplate to ACh, then more ACh could be released in the hypertonic solution without producing an obvious increase in amplitude. Nastuk and Parsons¹² studied the depolarisation produced by 0.011 mM carbamylcholine in normal Ringer and in Ringer plus 300 mM sucrose, and concluded that the sensitivity was unchanged. Another possibility is that the change in muscle fibre volume produces substantial changes in the cable properties of the endplate region. If this occurred, transient, identical pulses of ACh might generate the same conductance change and current flow at the endplate, and nonetheless give m.e.p.ps of different amplitudes because of altered cable properties. The initial experiments suggested that substantial changes in cable properties are unlikely, because the shape of the m.e.p.ps is so similar in the normal and in the hypertonic solutions. This was checked by eye and also by measuring the half-times for the decay of the m.e.p.ps (in the example shown in Fig. 1, normal Ringer = 13.3 ± 0.46 ms, $n = 22$; hypertonic Ringer = 13.2 ± 0.57 ms, $n = 29$).

This possibility was tested further by using the two micro-electrode method for voltage clamping the endplate, so that the amplitudes of the miniature endplate currents (m.e.p.cs) could be measured¹³. The disadvantage of this method is that there is a somewhat greater delay between the solution change and the reinsertion of two electrodes than in the previous experiments; in other respects the protocols were identical.

Figure 2 shows m.e.p.cs at various holding potentials at the same endplate in normal and in hypertonic solution. The m.e.p.cs have a slightly lower mean amplitude in hypertonic than in normal Ringer. (This result might be anticipated because the muscle fibre will also shrink in the hypertonic solution, increasing $[K]_{in}$ and $[Na]_{in}$, thereby making the reversal potential for the e.p.p. somewhat more negative. But this explanation glosses over a complicated series of possibilities. For example, the change in intracellular ion concentrations will change the surface potential on the inner side of the sarcolemma¹⁴, and this would be expected to change membrane ionic conductances.) The important point is that there was no indication of even a transient increase in the m.e.p.cs following the transition from hypertonic to normal Ringer, nor was there any notable change in the time course of the m.e.p.c. Similar results were obtained in two additional voltage clamp experiments.

Thus, abrupt change in tonicity, which must produce almost stepwise alterations in nerve terminal volume, do not change m.e.p.p. amplitudes or m.e.p.cs in the direction expected if the amount of ACh released per quantum depends on the concentration of free ACh in the cytoplasm of the terminal. From the present experiments it is still conceivable that quantal size depends upon free ACh, but this requires that following a substantial change in concentration—probably at least two-fold

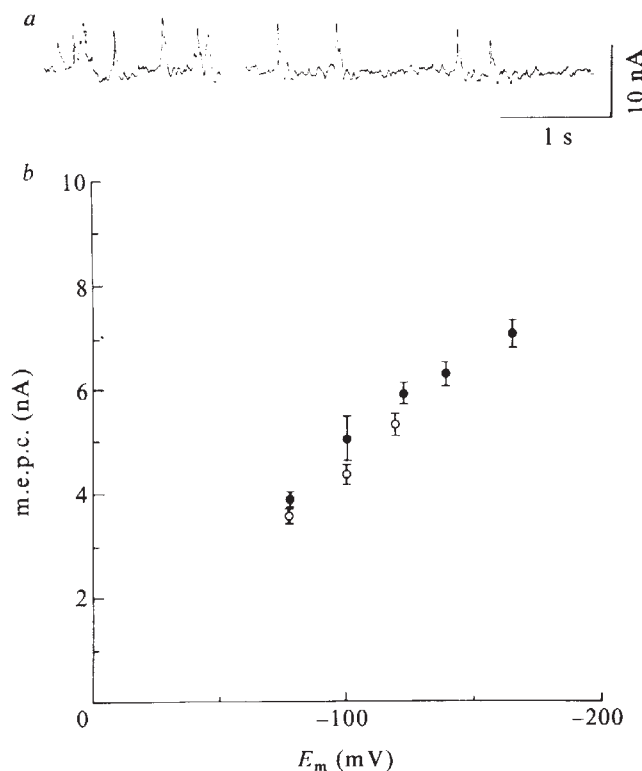


Fig. 2 *a*, Examples of the m.e.p.cs in Ringer (right hand trace) and in Ringer plus sucrose (left hand trace) at a holding potential of -121 mV. The solutions and the method of data collection are given in the legend to Fig. 1. *b*, M.e.p.cs recorded in a preparation in Ringer (●) and in Ringer plus 500 mM sucrose (○). The vertical bars indicate $\pm$ the s.e.m.

—the terminal is capable of readjusting to normal levels of ACh within one or two minutes. When these observations are taken together with the evidence that quantal size does not vary with changes in the membrane potential of the nerve terminals¹⁵, as would be expected if free ACh were released as a cation through a channel, then the balance is overwhelmingly in favour of a release mechanism which does not depend upon the free ACh concentration. The vesicle hypothesis, with suitable modifications to account for such observations as the preferential release of newly synthesised transmitter, offers a most reasonable alternative.

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Benzodiazepines specifically modulate GABA-mediated postsynaptic inhibition in cultured mammalian neurones

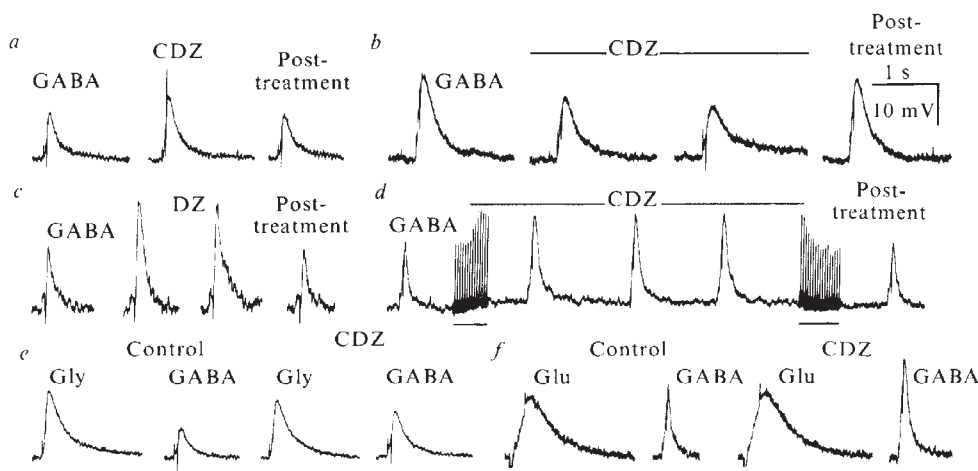
BENZODIAZEPINES (BDZ) such as diazepam (DZ) and chlordiazepoxide (CDZ) are commonly used anticonvulsants, muscle relaxants, antianxiety agents and hypnotics. The cellular mechanisms underlying these clinically important effects have not been established, but attention has been focused on the effect of BDZ on pre- and postsynaptic responses to the putative neurotransmitter amino acids, specifically glycine (Gly)^{1,2} and γ -aminobutyric acid (GABA)³⁻⁷. An effect of BDZ on postsynaptic Gly receptors was suggested by the finding that BDZ reduce strychnine binding to central nervous system (CNS) synaptic membrane fractions *in vitro*², but physiological studies, *in vivo*, have provided little support for such an interaction with Gly³. BDZ have been reported (1) to facilitate presynaptic inhibition in the spinal cord^{4,5}, at least partially GABA-mediated⁸⁻¹⁰; (2) to facilitate GABA-mediated pre- and postsynaptic inhibition in the cuneate nucleus⁶; (3) to mimic the presynaptic action of GABA on preganglionic nerve terminals¹², and (4) to antagonise^{13,14} and to enhance^{15,16} the postsynaptic action of GABA on CNS neurones. It has also been suggested that BDZ alter GABA metabolism^{4,5} or mobilise GABA from neuronal storage sites¹⁷, and BDZ have been demonstrated to bind specific receptors on synaptosomal membrane preparations derived from rat brain¹⁸, an effect which is independent of amino acid receptors. Thus, it is uncertain whether BDZ action is GABA or Gly receptor-mediated, due to an alteration of GABA metabolism, or due to direct binding to a specific receptor which binds BDZ. We have studied the effect of DZ and CDZ on amino acid-mediated postsynaptic responses in cultured mammalian spinal cord neurones and report that the BDZ selectively modify GABA-mediated postsynaptic inhibition in a dose-dependent fashion, augmenting the response at low doses and antagonising the response at higher doses.

Cultures were prepared from spinal cords removed from 13-14-d-old foetal mice as described previously¹⁹, and were grown on collagen-coated 35-mm dishes for 5-8 weeks before the study. Electrophysiological recordings were made on a

modified stage of an inverted phase microscope at 37 °C. All recordings were made in normal growth medium (90% minimal Eagle's medium/10% horse serum) to which MgCl₂ was added (final Mg²⁺ concentration of 10 mM) to suppress spontaneous activity and allow a clearer recording of postsynaptic responses. BDZ and amino acids were iontophoresed using a constant current device and high resistance θ -tube micropipettes (50-100 M Ω) filled with GABA (0.5 M, pH 3.5), Gly (0.5 M, pH 3.5), glutamate (Glu) (0.5 M, pH 8.6), (all from Sigma), DZ (10 mM, pH 3.5) and CDZ (100 mM, pH 3.5) (Hoffman-Roche, Nutley, New Jersey).

Both CDZ (Fig. 1a, d) (15 cells) and DZ (Fig. 1c) (6 cells) rapidly and reversibly augmented the response elicited by the iontophoresis of GABA (Fig. 1d). This augmentation could frequently be produced by simple diffusion of BDZ from the pipette tip but usually required passage of 1-5 nA of positive current. When augmentation was achieved, if additional current (1-10 nA) was passed through the BDZ pipette, antagonism of the GABA response was produced (Fig. 1b). The entire range of augmentation and inhibition was usually obtained without any alteration in resting membrane potential or input resistance, but a direct effect of BDZ was occasionally obtained (five cells), consisting of depolarisation and increase in conductance (not illustrated). The effect of these agents was specific for GABA since the responses to Gly (Fig. 1e) and Glu (Fig. 1f) were not augmented by CDZ application despite clear augmentation of GABA responses (five cells). The nature of this action of BDZ on the GABA response is unclear. One possible mechanism for augmentation of the response could involve an inhibition of the uptake of GABA, thus prolonging GABA synaptic action. This is unlikely as such an effect would be expected to prolong the time course of the response and this does not occur either with augmentation (Fig. 2a) or with antagonism (Fig. 2b), and would not explain the high dose antagonism of the response. The specificity of the interaction between BDZ and GABA suggests, however, that a direct effect of BDZ on the Cl⁻ conductance mechanism used by GABA and Gly²⁰ is unlikely; rather, the most likely site of action is at the GABA receptor with a modification of the affinity of the receptor for GABA. Such a conclusion is supported by initial analysis of the augmenting effect of CDZ on GABA dose-response curves; this effect is associated with a parallel shift to the left without alteration of the V_{max} para-

Fig. 1 Effect of benzodiazepines on amino acid responses in cultured mammalian spinal cord neurones. Intracellular recordings were made using 3M KCl micropipettes (25-40 M Ω) with the membrane potential hyperpolarised to -90 mV. Amino acid responses were elicited with 50 ms pulses of appropriate polarity while BDZ were applied with a steady cationic current. Under these conditions, the reversal potential for all amino acid responses is more positive than -30 mV, and thus all responses are depolarising. CDZ [as] and DZ [cs] reversibly augment the GABA response at low iontophoretic currents and reversibly depress the GABA response at higher currents [bs] (illustrated for CDZ only). A continuous record of augmentation of GABA responses by CDZ is shown in d. The penwriter is slowed (see bars below d) at onset and offset of CDZ current (time calibration bar, 5 s). The response to Gly (e) and Glu (f) are not augmented by CDZ despite clear augmentation of the GABA response. Calibration bars apply to all penwrite traces except as described in d.



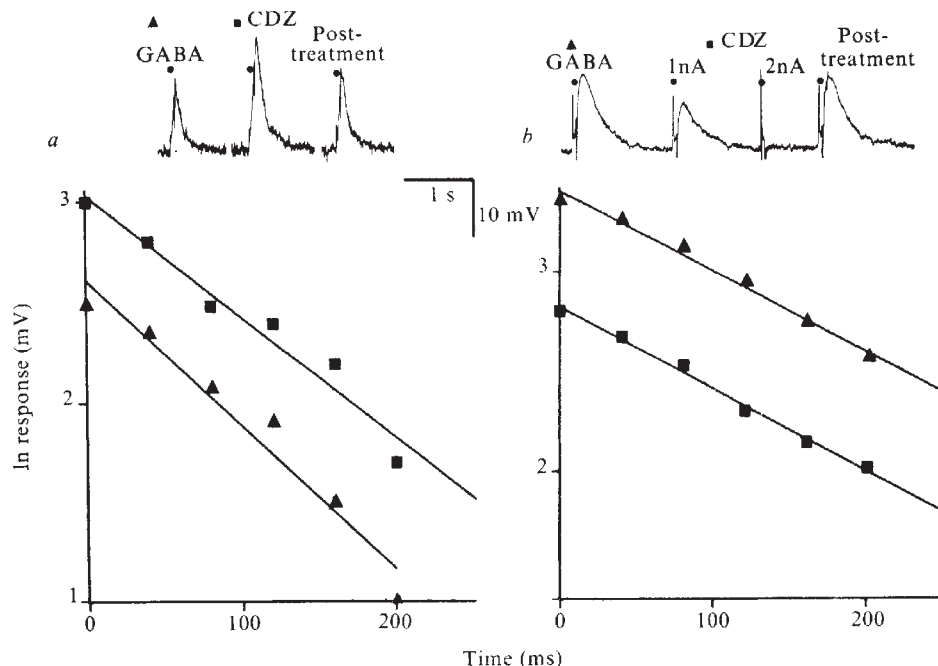


Fig. 2 Time course of GABA responses modulated by benzodiazepines. The effect of CDZ on the GABA response at low dose (a) and at higher dose (b) did not affect the time course of the GABA response as shown in semi-logarithmic plots of the amplitude of the GABA response following the peak response as a function of time.

meter in the Lineweaver–Burke plot (unpublished observations). Modification of coupling between receptor and ionophore is also a possible explanation; an effect on channel duration would be unlikely since this would modify the overall time course of the response, but an effect on unitary channel conductance could be a possible mechanism. Insufficient data is available to allow interpretation of BDZ antagonism of GABA responses.

Thus, this study directly demonstrates that the BDZ have a specific yet dose-dependent effect on GABA-mediated postsynaptic inhibition in mammalian spinal cord neurones, with augmentation obtained at low doses and depression elicited with higher doses. The results do not support BDZ enhancement of glycine-mediated postsynaptic inhibition, as has been suggested previously^{1,2}. Furthermore, the dual effect of BDZ on GABA responses may help to explain why previous studies on different preparations have found opposing results^{13–16}. Augmentation of GABA-mediated pre- and postsynaptic inhibition may be the basic feature of its anticonvulsant activity since augmentation of GABA-mediated postsynaptic inhibition by the anticonvulsant phenobarbital has been observed in tissue cultured mammalian neurones^{20,22}, while diphenylhydantoin has been reported to prolong GABA responses in invertebrates²³. The therapeutic role of the inhibition of GABA responses by BDZ is not clear, but it may be responsible for some of the toxic side effects of CDZ and DZ such as ataxia, seizures and tremor. The role of the direct effect of BDZ on neuronal membrane properties seen in these experiments and in sympathetic preganglionic nerves^{11,12} and that of direct BDZ binding to specific receptors derived from rat brain¹⁸ is also uncertain. In the present study, the direct effects were observed at BDZ currents higher than those required for GABA response augmentation. Thus, if GABA response enhancement underlies its anticonvulsant action, the direct effects of BDZ may be relevant only at toxic or non-therapeutic doses.

A study published after submission of our manuscript showed that CDZ selectively augments GABA responses elicited from neurones in chick spinal cord cultures²⁴.

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Isolation of specific neurones by affinity methods

THE complex heterogeneity of cell populations in the nervous system severely limits the study of many important questions in neurobiology. Improvement of the current techniques of neuronal cell culture will require methods for selective isolation of neurones belonging to specific functional classes. Present methods include a degree of separation of neurones from non-neuronal cells by physical methods^{1–3} but these differentiate only on the basis of size or density. The separation of classes of tumour cells and lymphocytes has been accomplished by affinity chromatography using lectins or antibodies against surface antigens^{4–7}. In principle neurone purification could be approached in the same way. Among other surface identities,

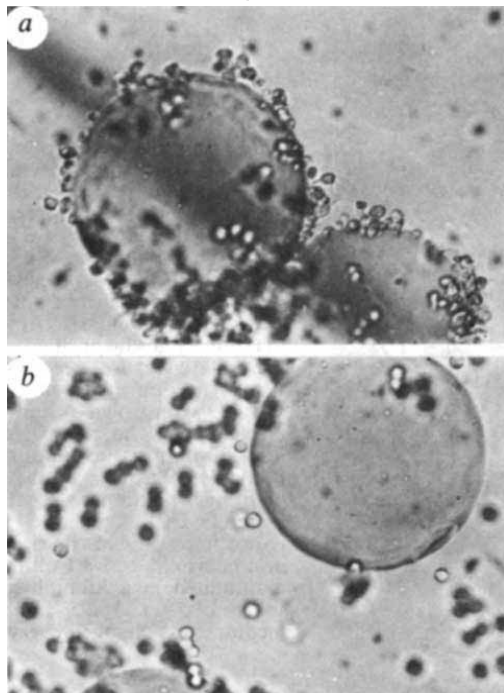


Fig. 1 *a*, Chick embryo sympathetic ganglion neurons bound to α BT-Sephadex 6MB beads. $\times 120$. *b*, The same beads do not bind HeLa cells significantly. α BT-derivatised beads were synthesised as follows; 100 μ l of *p*-aminobenzoylhexylamino Sephadex 6MB¹² beads were diazotised in 0.1 M sodium nitrite in 0.1 M HCl-0.2 M NaCl for 10 min at 0 °C. The beads were washed with ice-cold PBS (after excess nitrous acid had been destroyed with 0.1 M sulphamic acid) and added immediately to a solution of approximately 1 mg of α BT in 0.5 ml of 0.05 M borax pH 8. Reaction was allowed to proceed overnight at 4 °C and the resulting dark-yellow beads were washed extensively with PBS. The amount of toxin covalently attached to the beads was estimated by measuring the A_{280} of the supernatant at the end of the reaction. Typically a concentration of α BT on the beads of approximately 1 mM was achieved.

neurons have neurotransmitter receptors which provide one basis for functional classification and potentially provide targets for affinity probes of high specificity. As a model system we chose the chick embryo sympathetic ganglion neurons which are known to possess high numbers of the nicotinic type of acetylcholine (ACh) receptor⁸. These cells also possess high numbers of receptors for α -bungarotoxin

(α BT)⁹. We report here the successful separation by affinity chromatography of the principal neurones from sympathetic ganglia using α BT as a probe.

α BT was attached to Sephadex 6MB by reacting the toxin in borax buffer pH 8 with diazotised *p*-aminobenzoylhexylamino Sephadex 6MB (ref. 10). This procedure results in the formation of an azo linkage with one or other of the two histidine residues (positions 4 and 67)¹¹. In view of the structural similarity between types I and II neurotoxins¹¹ and the fact that the extra disulphide bond in type II toxins is not essential for activity¹² it is reasonable to assume a conformation for α BT similar to that recently determined for erabutoxin b by X-ray crystallography¹³. In this conformation the two histidines in α BT are well removed from the active site and their modification is unlikely to affect the biological activity. Indeed the derivatised beads bind chick embryo sympathetic ganglion neurones in preference to other cell types (Fig. 1).

Sympathetic ganglion cells were prepared from 19-d chick embryos as previously described¹⁴. At this age the neurones have approximately 2×10^5 α BT receptors per cell⁹. After trypsinisation (0.025%) the cells were dissociated in Puck's saline G containing 2 mg ml⁻¹ bovine serum albumin, 20 μ g ml⁻¹ deoxyribonuclease (DNase) and 10 units ml⁻¹ nerve growth factor (NGF)¹⁵; this will be referred to as medium A. The cells were then filtered through nylon gauze (pore size < 50 μ m), centrifuged and resuspended at 1×10^6 cells ml⁻¹ in medium A. The α BT-derivatised macrobeads were held in the bottom of a small glass water-jacketed column by a piece of nylon gauze held in place by a tightly fitting Teflon ring. The assembled column without beads was autoclaved and all manipulations were carried out in a sterile laminar flow cabinet. The beads were extensively washed with sterile phosphate-buffered saline (Ca²⁺ Mg²⁺-free Dulbecco's PBS), then medium A. The flow rate was adjusted to 1–2 ml h⁻¹ and the cell suspension loaded. It was important to maintain a temperature of 0–4 °C throughout the isolation procedure for optimal viability of the neurones. The beads were washed until very few non-neuronal cells were present in the eluate. After further washing with PBS containing DNase (4 μ g ml⁻¹) and subsequently with PBS containing DNase and 0.1% trypsin, the column was clamped off, the temperature raised to 37 °C and incubation allowed for 2–3 min. Foetal calf serum (FCS) was then added and the cells eluted with about 10 ml of F15 medium (GIBCO) containing 5% FCS (growth medium) into a sterile centrifuge tube. The cells were centrifuged, resuspended in 1 ml growth medium containing 10 U ml⁻¹ NGF and cultured.

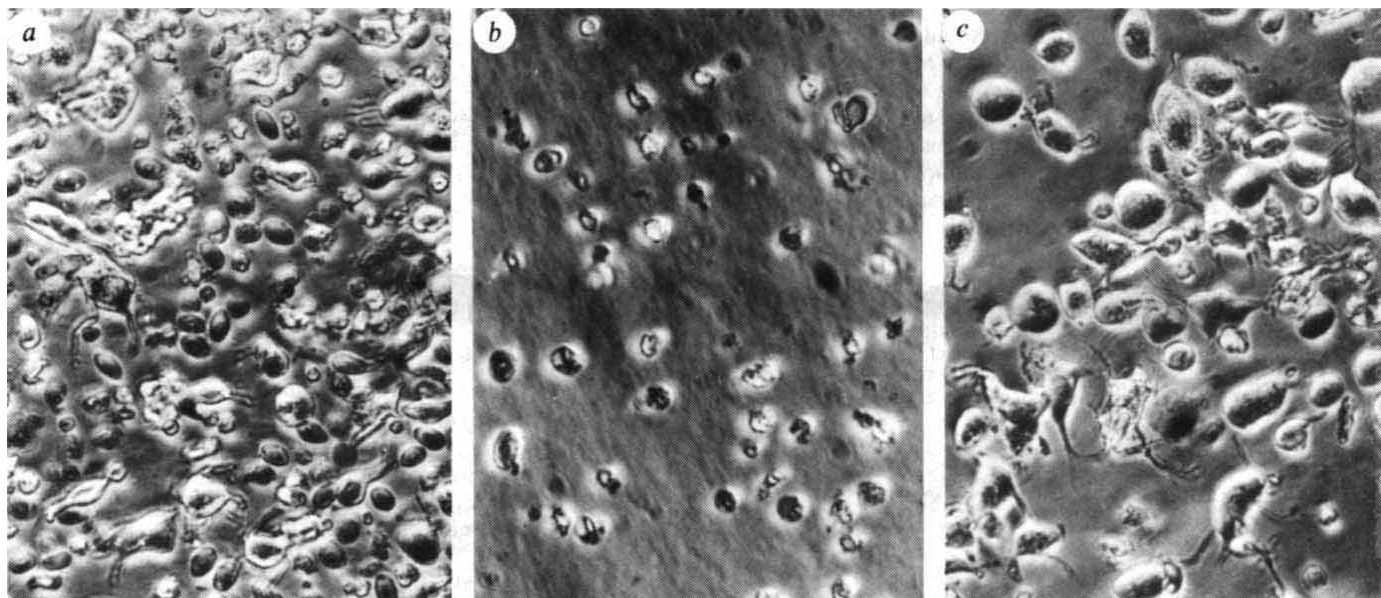


Fig. 2 Purification of sympathetic ganglion neurones. *a*, Original cell suspension containing approximately 10% principal ganglion neurones. *b*, Eluate from the affinity column during washing. Essentially all non-neuronal elements. *c*, Final suspension of purified neurones after removal from column by trypsin (0.1%). Phase contrast $\times 370$.

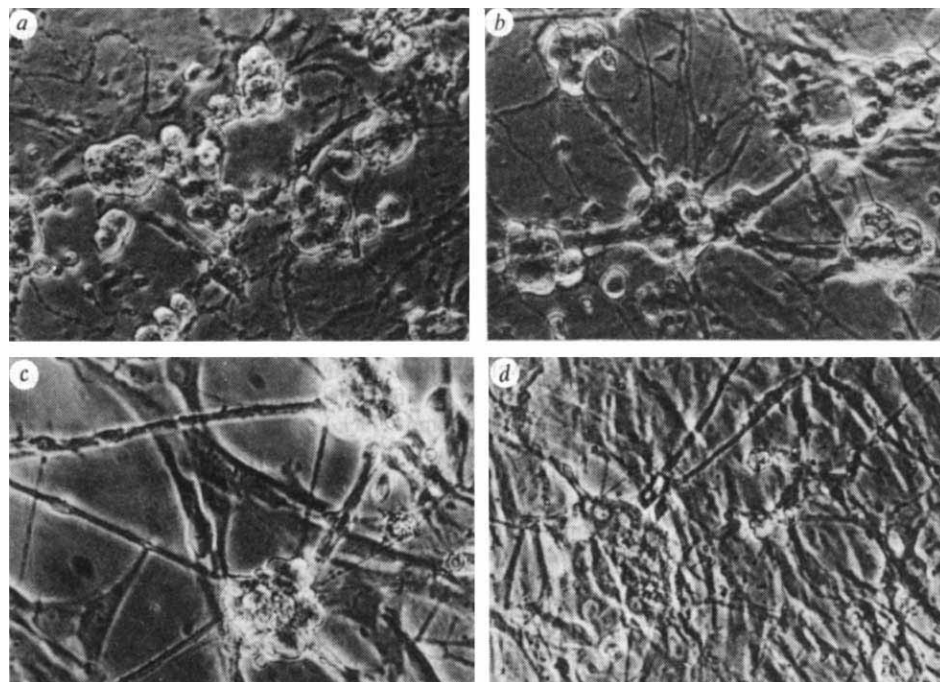


Fig. 3 Phase contrast photomicrographs of cultured sympathetic ganglion neurones: parallel purified and nonpurified cultures. $\times 328$. *a*, Purified neurones, 1 d *in vitro*. *b*, Purified neurones, 6 d *in vitro*. *c*, Non-purified neurones, 1 d *in vitro*. *d*, Non-purified neurones, 6 d *in vitro*. Comparison of (*b*) and (*d*) shows that the purified neurones are relatively free of fibroblasts while non-purified cultures are virtually overgrown by non-neuronal elements. The cells were plated into 33-mm tissue culture dishes (Kayline Plastics). For isolated neurones, plates were coated with tail collagen¹⁷. A collagen substrate is not necessary in the presence of non-neuronal cells. Cultures were maintained in a humidified atmosphere of 5% CO₂-95% air and the medium was changed every second day.

Figure 2 shows the cell suspension at different stages during the purification. The original cell suspension contained about 10% principal neurones and the final cell suspension > 95% neurones. Thus we have achieved a purification of the neurones of 9 to 10 fold. By the concomitant use of differential plating¹⁶ and the use of mitotic inhibitors for a short period after plating, the few remaining non-neuronal cells could be eliminated, giving 100% purity. Alternatively, nonspecific binding may be eliminated by more extensive washing or possibly by the inclusion of a small inert charged molecule to eliminate charge interactions.

The isolated neurones were viable after 6–8 h on the column. Cultured, purified neurones are compared with parallel non-purified cultures in Fig. 3. After the same period *in vitro* those neurones which had gone through the affinity column were still free of non-neuronal cells.

The functional viability of the isolated neurones was examined by electrophysiological measurements (Fig. 4). The neurones responded in an all-or-none fashion to electrical stimulation and responded to iontophoretically-applied acetylcholine, showing desensitisation on repeated application. Autoradiography showed that essentially all the isolated neurones bind ¹²⁵I- α -bungarotoxin (data not shown).

Thus, we have been able to obtain essentially pure, viable and electrically active neurones. The same results were obtained using as an affinity probe the α -neurotoxin from the venom of *Naja nigricollis* purified by CM cellulose chromatography. The availability of suitable specific immobilised ligands for other surface receptors would open up possibilities for in-

vestigation *in vitro* of important questions such as synaptic specificity, neuronal-non-neuronal cell interactions and the behaviour of particular neurone populations in certain disease states. We are investigating the possibility of using small molecule affinity probes and applying our isolation procedure to neurones from the brain.

We thank John Leah for carrying out the electrophysiological measurements. This work was supported by the Australian Research Grants Committee.

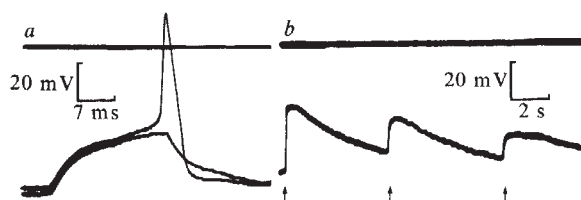
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Fig. 4 Intracellular recordings from purified chick embryo sympathetic ganglion neurones, 6 d *in vitro*. *a*, All-or-none response to a depolarising current pulse of 0.2 nA. *b*, Response to successive iontophoretic applications of acetylcholine (arrows). Ejecting current 75 nA, 70 ms. Electrical activity was monitored as previously described¹⁴.



Paired helical filaments of the Alzheimer type in cultured neurones

ALZHEIMER'S disease, a common progressive dementia of later life, is associated with several histopathological changes. Although one of these pathological changes, neuritic plaques with amyloid cores, has been reproduced in brain of mice following inoculation with scrapie agent^{1,2}, Alzheimer-type neurofibrillary degeneration has not yet been reproduced in the laboratory. This unique form of human neurofibrillary degeneration is composed of arrays of paired 100-Å diameter filaments twisted into a helical configuration with a period of approximately 800 Å (refs 3, 4). We present here evidence that paired helical filaments

morphologically closely resembling those found in the human disease are induced in cultured human foetal cerebral cortical neurones after exposure to an extract prepared from Alzheimer-affected brain.

Explanted fragments (0.5 mm^3) of cerebral cortex derived from two human foetuses of approximately 12–14 weeks gestation were used in this initial study. Explant cultures were maintained on collagen-coated coverslips⁵ in a medium composed of 35% Simm's balanced salt solution, 40% heat-inactivated human cord serum and 25% medium 199 (GIBCO), with 600 mg% glucose (final concentration). The coverslips with cultures were placed into plastic Petri dishes (Falcon) and incubated at 36°C in a water-saturated atmosphere of 95% air and 5% CO_2 . The culture medium was changed twice weekly. A saline extract was prepared from cerebral cortex of a patient who died aged 63 after a 6-yr history of progressive intellectual deterioration and whose brain exhibited widespread neurofibrillary degeneration and senile plaques typical of Alzheimer's disease. The brain was removed 12 h post-mortem and stored frozen at -90°C . Four months later a cortical sample of approximately 2 cm^3 was removed from the tip of the temporal lobe, thawed and homogenised in an equal volume of sterile physiological saline at 4°C in a Potter–Elvehjem homogeniser. The homogenate was spun in a bench-top centrifuge at 1,500 r.p.m., the pellet discarded, and the cell-free supernatant passed through a Millipore filter of 4,500 Å pore size. Ultrastructural examination of the filtrate by standard positive and negative staining methods did not reveal fragments of paired helical filaments. The filtrate did, however, contain fragments of microtubules, ranging in length from 600 to 2,300 Å and small membrane fragments. No particles

Fig. 1 Electron micrograph of an unaffected human foetal neurone representative of the culture system used in the study; 35 d *in vitro*. Arrowhead identifies presynaptic terminal of an axosomatic contact. Scale bar, $0.5 \mu\text{m}$.

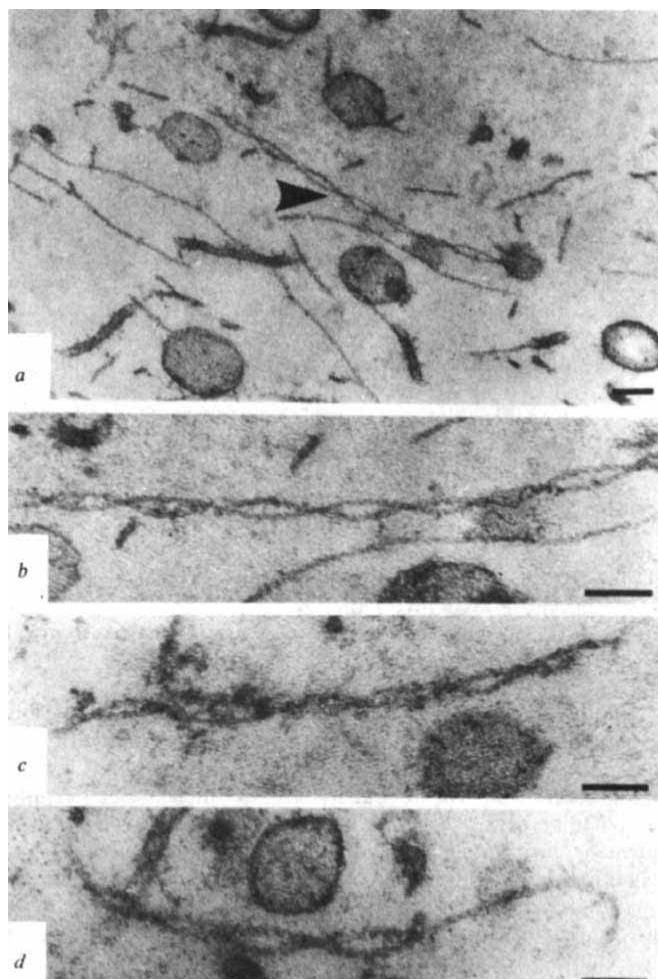
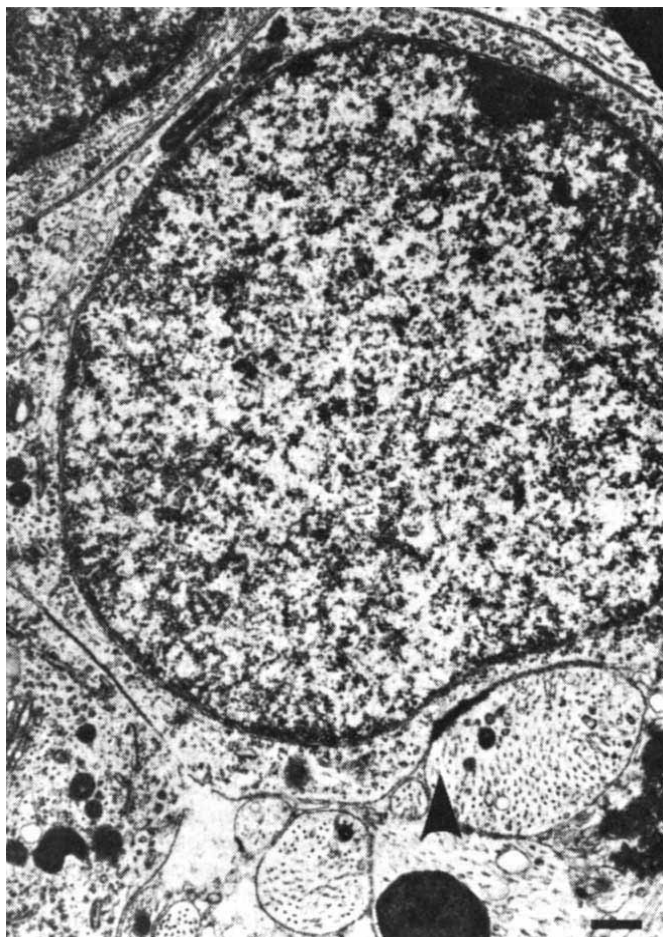


Fig. 2 Electron micrographs of paired helical filaments observed in foetal brain cultures exposed to an extract of Alzheimer affected brain; 35 d *in vitro*. *a*, Low power aspect of process with paired helical filament (arrowhead), enlarged in *b*; *c*, *d*, additional examples. Scale bars, $0.1 \mu\text{m}$.

exhibiting viral morphology were encountered. The human, foetal cerebral explants were exposed to an approximately 5% solution of the Alzheimer brain extract for the first 6 d in culture. After this initial exposure, feeding was carried out with pure culture medium. The cultures were fixed at various intervals after inoculation with 3.5% glutaraldehyde in phosphate buffer.

Detailed ultrastructural examination of cultures fixed after 4, 8 and 11 d of incubation revealed no paired helical filaments and the morphological characteristics of the cells were identical to those in control cultures (Fig. 1). However, after 14 d incubation, occasional paired helical filaments were observed, and after 35 d of incubation, paired helical filaments were found in many cell processes. Approximately 3% of all cell processes ($n = 491$) at 35 d incubation contained one or more paired helical filaments. Synaptic contacts established that many of the processes containing these paired filaments were definitely of neuronal origin. Many examples of paired helical filaments occurred in cells in which the cytoplasm seemed swollen and the density of organelles was reduced. The plasma membranes of affected cells were intact, although microtubules seemed fragmented and mitochondria were often dense. Many of the paired helical filaments seemed to be assembled from separate, morphologically normal appearing neurofilaments (Fig. 2*d*). The average diameter of the individual filaments composing the helical structures in the cultured cells was 95 Å (s.d. 16 Å , $n = 12$) and the average width of pairs was 289 Å (s.d. 45 , $n = 13$). This is to be compared with the

paired helical filament in Alzheimer's disease in which individual filaments have an average diameter of 95 Å (s.d. 17 Å, $n = 13$) and a mean overall width of 241 Å (s.d. 55, $n = 9$). The paired, helical filament of Alzheimer's disease usually exhibits nodes with a mean period of approximately 800 Å, although internode distances of up to 1,000 Å are not uncommon⁶. In the cultured foetal cells, the internode period ranged from 800 to 1,600 Å, and a mean value of 846 Å (s.d. 133 Å) was measured on several typical paired helical filaments displaying up to 10 nodes (Fig. 3c). In contrast to the neurofibrillary tangle of Alzheimer's disease, in which dense aggregates of paired helical filaments occur, the number of paired helical filaments per cultured cell was small, and dense aggregates were not observed. Typically, the paired helical structures induced in culture did not display electron dense material along the filaments and seemed more distinct than those observed in Alzheimer's disease.

Electron microscopic examination of over 500 human cerebral cultures not exposed to the Alzheimer extract has never revealed paired helical filaments, although these cultures were exposed to a variety of toxic agents, including aluminium⁷, osmotic stress, fungi and bacteria. Moreover, over 500 brain explants from goldfish⁸, foetal rabbits and foetal mice similarly exposed to various toxic agents never exhibited paired helical filaments.

The observations described here suggest at least three possibilities. The Alzheimer donor brain may have contained metabolic products, possibly associated with the degeneration, which are capable of assembling neurofilaments into paired, helical structures. Such molecules may be postulated to have high affinities for specific sites on normal neurofilaments, resulting in their cross bonding and twisting into a helical configuration. Alternatively, the assembly factor may arise from a viral product present in brain affected by Alzheimer's disease, or, a viral-like agent may have been transferred to the cultured cells and the

paired helical filaments may have resulted from the replication of a transmissible agent in the cultured human brain cells. Although several morphologically distinct types of viral particles were observed in the cultures exposed to the extract from Alzheimer-affected brain their relationship to the paired helical filaments is not yet known.

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Note added in proof: Since the introduction of Alzheimer material into the incubator, repetition of the above experiments has revealed the presence of an occasional control explant with paired helical filaments. Since all other variables have remained unchanged, we interpret this as support for the possibility that paired helical filaments are a response of human neurones to an infectious agent.

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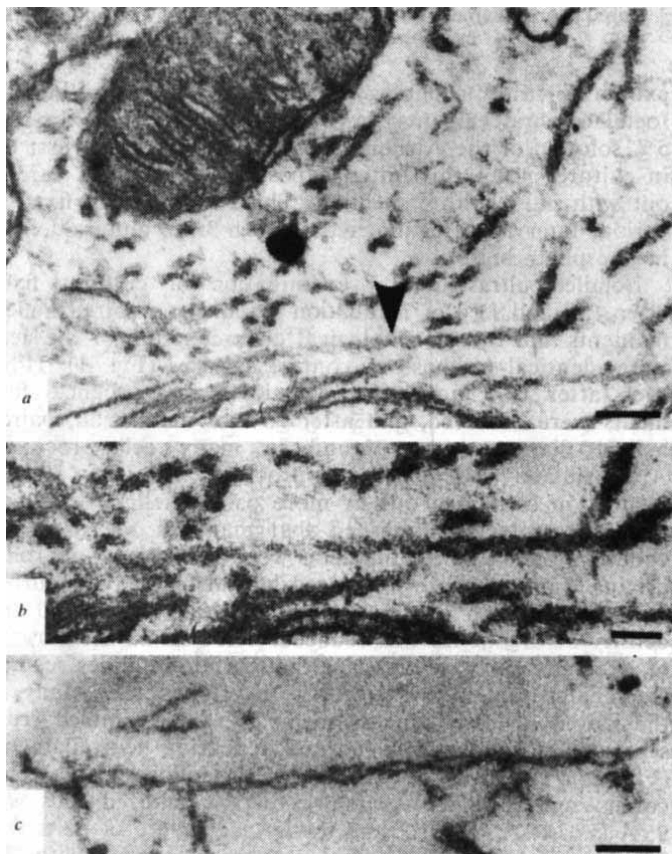
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Identification of the binding protein which may be the target of penicillin action in *Bacillus megaterium*

PENICILLINS and cephalosporins (β -lactam antibiotics) are believed to kill bacteria by inhibiting reactions involved in the final stages of cell wall assembly¹. It is widely assumed that inhibition of a transpeptidase involved in the insertion of nascent peptidoglycan strands into the cell wall directly leads to cell death. One approach to the study of the lethal action of penicillin has involved the investigation of penicillin-binding proteins (PBPs) which, with few exceptions, are located exclusively in the cytoplasmic membrane of bacteria^{2–4}. PBPs form covalent complexes with benzyl-(¹⁴C)-penicillin, and can be detected by fluorography after sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. All species of bacteria examined contain multiple PBPs. We have shown that the isolated membranes of *Bacillus megaterium* contain five proteins which bind benzylpenicillin covalently and we have measured their relative affinities for the antibiotic⁴. However, the accessibility of the PBPs and their affinities for penicillin may be different in whole cells, and to identify the PBP which represents the lethal target for benzylpenicillin, it is necessary to investigate the binding of the antibiotic to the PBPs of whole cells. We report here that only one of the five PBPs in *B. megaterium* reacts with benzylpenicillin to any significant extent at concentrations just sufficient to kill the cells, and that the characteristics of the inhibition and of the breakdown of the PBP-penicillin complex are consistent with its being the protein which catalyses transpeptidation.

It is difficult to run quantities of whole cell protein sufficient to reveal the PBPs by fluorography in a reasonable time, without overloading the polyacrylamide gel. Because all the PBPs of *B. megaterium* are located in the membrane, it was possible to carry out the experiment by incubating intact cells with penicillin followed by preparation of the membrane fraction and examination of the PBPs. A preliminary investigation showed that release of bound penicillin from the PBPs was slow, with PBPs 1 and 5 having the shortest half-lives of breakdown at

Fig. 3 Electron micrographs comparing paired, helical filaments from an Alzheimer affected brain (*a*, *b*) to a paired, helical filament in a cultured foetal brain cell; 35 d *in vitro*. In (*a*), (*c*), scale bar 0.1 μ m; *b*, 0.05 μ m.



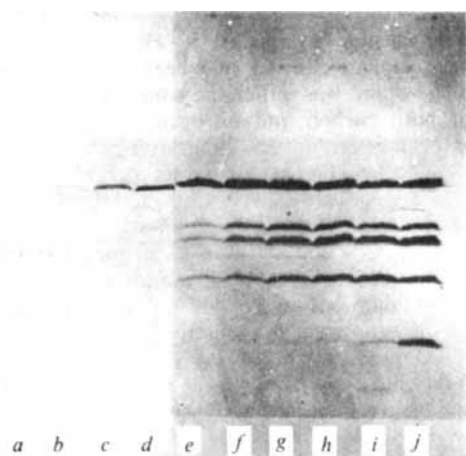


Fig. 1 Fluorogram demonstrating the sensitivity of membrane-bound penicillin-binding proteins in whole cells of *Bacillus megaterium*. 10-ml cultures of *B. megaterium* KM growing exponentially in a complex medium were treated with a range of concentrations of benzyl-(^{14}C)-penicillin (51 Ci mol^{-1}) when the cell density had reached $0.3 \text{ mg dry weight ml}^{-1}$. After 5 min at 37°C , 10 mM nonradioactive benzylpenicillin was added, the cells were collected rapidly by centrifugation at 4°C ($27,000g$, 1 min), washed in ice-cold 25 mM Tris-HCl, 5 mM MgCl_2 , pH 7.2, resuspended in 0.3 ml of the same buffer containing lysozyme ($250 \mu\text{g ml}^{-1}$) and DNA-ase ($5 \mu\text{g ml}^{-1}$), and incubated at 37°C until lysis was complete (5 min). The membranes were collected by centrifugation at $40,000g$ for 20 min and dissolved by boiling for 1 min in 50 μl buffer containing 10% glycerol, 1% SDS, 1% 2-mercaptoethanol and 0.002% bromophenol blue in 10 mM Tris-HCl, pH 7.2. The membrane proteins were separated by electrophoresis on a 10% polyacrylamide slab gel containing SDS (ref. 5), and the penicillin-binding proteins (PBPs 1–5, numbered in order of decreasing molecular weight, ref. 4) revealed by fluorography^{6,7}. The following concentrations of benzyl-(^{14}C)-penicillin were employed ($\mu\text{g ml}^{-1}$): a, 0.003; b, 0.006; c, 0.01; d, 0.03; e, 0.06; f, 0.1; g, 0.3; h, 0.6; i, 1.0; j, 5.0.

37°C (15–40 min). The conditions of membrane isolation (5 min at 37°C with lysozyme, followed by the remainder of the manipulations being carried out at 4°C (approximately 45 min)) were such that they would not result in significant breakdown of PBP–penicillin complexes.

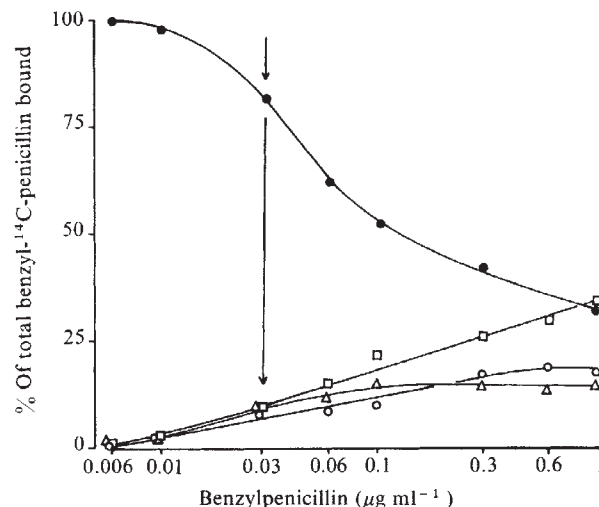
Whole cells of *B. megaterium* were incubated with a range of concentrations of benzyl-(^{14}C)-penicillin, collected, washed, lysed by treatment with lysozyme and the protoplast membranes obtained by centrifugation. The proteins of the membrane were fractionated by SDS-polyacrylamide slab-gel electrophoresis⁵ and the PBPs detected by fluorography^{6,7}. This enabled the affinities of the PBPs for benzylpenicillin in whole cells to be determined. The relative affinities of the PBPs were the same as had been found previously when isolated membranes were treated with benzylpenicillin⁴, although the absolute concentrations of the antibiotic resulting in 50% saturation of the PBPs were lower than when membranes were used. The PBPs are likely to be more accessible in membranes than in whole cells, and this suggests that the amount of penicillin available to react with the PBPs of membranes may have been a limiting factor at the lower concentrations of the antibiotic. This possibility will be considered elsewhere. It is clear from the results in Fig. 1 that PBP 1 has the highest affinity for benzylpenicillin, but this information alone is insufficient to implicate this protein as the lethal target of penicillin. It is necessary to investigate which PBPs bind penicillin when growing cells are labelled at concentrations of the antibiotic which are just sufficient to interfere with growth or cause cell death. The growth of *B. megaterium* as measured by increase in turbidity was inhibited by 50% at a concentration of $0.03 \mu\text{g}$ benzylpenicillin per ml; at this concentration, the saturation of PBPs 1–5 in whole cells was 75, 12, 8, 4 and 0% respectively. Over a long period, the inhibition of an essential enzyme by as little as 10% might be sufficient to impair growth, but in this short term growth experiment over a period of three generations,

it seems unlikely that such a degree of inhibition (of PBPs 2 and 3) would account for the immediate inhibition of growth. We therefore conclude that PBP 1 is most likely to be the lethal target of penicillin.

At the minimum growth inhibitory concentration of $0.03 \mu\text{g ml}^{-1}$, more than 80% of the total radioactivity bound to the PBPs was present in PBP 1, with approximately 5% on PBPs 2, 3 and 4 (Fig. 2). As the concentration of benzylpenicillin was lowered further to that which inhibited the growth of cells from a small inoculum ($10^6 \text{ cells ml}^{-1}$) in liquid medium ($0.01 \mu\text{g ml}^{-1}$), or that which inhibited the growth of single cells on agar ($0.006 \mu\text{g ml}^{-1}$), no radioactivity was found associated with PBPs 2–5, while appreciable binding of benzyl-(^{14}C)-penicillin to the PBP 1 of intact cells was still demonstrable (Fig. 1). This is further indication that PBP 1 is of prime importance in the interaction of benzylpenicillin with whole cells of *B. megaterium*.

It is possible that benzylpenicillin interacts non-covalently with the killing site, or forms a covalent complex which breaks down so rapidly before or during SDS gel electrophoresis that it is not subsequently detected as a PBP. Under these circumstances it would obviously not be possible to correlate the killing site of penicillin with a particular PBP. The characteristics of the interaction of benzylpenicillin with PBP 1, however, are identical to those of the inhibition of the natural peptidoglycan transpeptidation reaction which is catalysed by wall/membrane preparations of *B. megaterium*. This transpeptidation reaction is inhibited by 50% at $0.1 \mu\text{g}$ benzylpenicillin per ml (ref. 8), a concentration which results in approximately 50% saturation of PBP 1 in isolated membranes. Preliminary studies on the interactions of benzylpenicillin with the natural transpeptidation system suggest that a covalent complex is formed, and subsequently breaks down, with a half life of 50–60 min at 23°C . PBP 1 interacts with benzylpenicillin to form a covalent complex which also breaks down with a half life of 60 min at 23°C , and this is consistent with the hypothesis that the natural transpeptidation reaction which represents the lethal target of penicillin action is catalysed by PBP 1. Proof of the hypothesis will have to await the demonstration that purified PBP 1 can catalyse a transpeptidation reaction. PBP 1 has been solubilised and purified almost to

Fig. 2 Binding of benzyl-(^{14}C)-penicillin to the proteins of whole cells of *Bacillus megaterium*. The degree of blackening of the X-ray film resulting from the (^{14}C)-penicillin–protein complexes was determined by scanning the fluorogram shown in Fig. 1 with a Joyce-Loebl micro-densitometer and determining the areas of the peaks thus obtained by weighing. The amount of radioactivity bound to the individual PBPs at the various concentrations of benzyl-(^{14}C)-penicillin is expressed as % of the total radioactivity bound at that concentration. The arrow denotes the concentration of benzylpenicillin which just inhibits growth of the organism over a period of three generations (minimum growth inhibitory concentration). ●, PBP 1; △, PBP 2; □, PBP 3; ○, PBP 4.



homogeneity but no enzymic activity in peptidoglycan biosynthesis has yet been ascribed to it⁴. It will be necessary to devise natural model transpeptidation reactions that can be catalysed by the penicillin-sensitive enzymes involved in the terminal stages of peptidoglycan biosynthesis. Such reactions exist for *Salmonella typhimurium*, in which it has been shown that PBP 4 and probably PBP 1 catalyse transpeptidation⁹, and also for *Escherichia coli*¹⁰, but all attempts to develop similar systems for the Gram-positive bacilli have been unsuccessful.

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Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids

CONJUGATIVE plasmids of bacteria are extrachromosomal genetic elements able to bring about DNA transfer by conjugation. The best-studied system is that of F-like plasmids in *Escherichia coli*, where at least 12 genes are involved¹. Expression of the transfer system in this case is subject to negative control^{2,3}. We report here that the newly discovered conjugative activity of Ti plasmids is inducible by specific amino acid derivatives that are also the substrates for plasmid-coded catabolic enzymes. This finding further extends the analogy between the regulation of the lactose operon and of transfer functions.

The Ti plasmids confer oncogenicity to *Agrobacterium tumefaciens* strains responsible for the crown-gall disease of plants^{4–7}. The plasmids apparently fall into three classes, between which there is only a limited degree of homology^{7–11}. First, octopine Ti plasmids trigger octopine (N-2-(D-1-carboxyethyl)-L-arginine) synthesis in transformed plant cells. Such plasmids also confer to these bacteria the ability to utilise octopine as sole carbon and/or nitrogen sources. All the octopine Ti plasmids isolated thus far from different strains seem to be nearly, if not completely, identical^{7–11}. Second, nopaline Ti plasmids trigger nopaline (N-2-(D-1-3-dicarboxypropyl)-L-arginine) synthesis in transformed plant cells. They also confer to their bacterial hosts the ability to use nopaline as sole carbon and/or nitrogen sources. The nopaline Ti plasmids isolated from different strains show a wider range of relatedness^{7–11}. The third kind of Ti plasmids has, so far, no recognised property other than oncogenicity.

We have shown that the Ti plasmids are conjugative^{12,13} as they can be efficiently transferred to non-oncogenic recipient strains of *Agrobacterium* in certain conditions. In our previous work we made use of the fact that these plasmids are catabolic plasmids to select for transconjugants. We noted that plasmid transfer could only be observed if the crosses had been performed in the presence of the arginine derivative—an 'opine'—that is specific for the type of Ti plasmid used in the transfer experiment. One possible interpretation for this result was that octopine or nopaline

induces the conjugative activity of octopine Ti plasmids and nopaline Ti plasmids respectively. However, as the mixed donor and recipient cultures had been inoculated on media in which the opine was either the source of nitrogen or the source of both carbon and nitrogen, some selection must also have been operating to favour transconjugants relative to recipient cells. The experiments described here were designed to distinguish between the models of induction of transfer and selection of transconjugants.

The purpose of a first series of experiments was to determine whether incubation of the donor cells in an opine-containing medium before mixing with the receptor resulted in the induction of conjugative activity. Octopine was found to induce conjugative activity of the Ti plasmid of strain B6S3 (Table 1). Crosses performed with donors that had been previously cultured on octopine yielded transconjugants whereas donors cultivated on arginine and pyruvate (the immediate products of octopine catabolism¹⁴) did not transfer their plasmid in the absence of octopine. When the filters with the mating mixture were incubated on arginine and pyruvate medium, relatively low frequencies of transconjugants were obtained; however, when octopine was used instead of arginine and pyruvate in the mating medium, high frequencies of transconjugants were observed. Their increased frequency is probably due to the duration of the incubation of the mating mixture (48 h) in the presence of octopine; this probably resulted in an increased number of donors transferring their Ti plasmid and also an enrichment of transconjugants relative to the recipients, since octopine was the sole carbon and nitrogen source in the mating medium. Two other opines, lysopine and octopinic acid, which are synthesised concomitantly with octopine in crown-gall cells¹⁵ and can also be utilised as both carbon and nitrogen source by *Agrobacterium* strains harbouring an octopine Ti plasmid, also induce conjugation, but to a lesser degree than octopine. In contrast histopine, a novel

Table 1 Induction of plasmid transfer

Mating medium	Preculture medium (C and N sources)	
	Arginine + pyruvate*	Octopine
Arginine + pyruvate	$\leq 10^{-7}$	7×10^{-5}
Octopine	2×10^{-3}	8×10^{-3}

Values shown represent transconjugant frequencies amongst the recipients. The donor strain B6S3, which carries an octopine Ti plasmid, was crossed with the recipient strain C58C1. Single colony isolates of B6S3 were grown in liquid minimal medium containing KH_2PO_4 $8.0 \times 10^{-2}\text{M}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ $6.5 \times 10^{-4}\text{M}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ $7.0 \times 10^{-3}\text{M}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ $1.7 \times 10^{-3}\text{M}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ $1.0 \times 10^{-5}\text{M}$, pH adjusted to 7.0 with KOH 6N, supplemented with either octopine 10 mM or arginine 10 mM plus pyruvate 10 mM as carbon and nitrogen sources. The cultures, in exponential phase of growth, were mixed in a ratio of 10:1 with recipient C58C1 cells exponentially growing on arginine plus pyruvate minimal medium. The bacterial mixtures were diluted to A_{680} of 0.2 and 5-ml samples were forced through Millipore filters (pore size 0.45 μm). The filters were incubated for 48 h at 25 °C on agar (1.5%) minimal medium (mating medium) supplemented with 10 mM octopine, or arginine plus pyruvate. The filters were introduced into test tubes containing 5 ml of sterile water and the bacteria on the filters were suspended by vortex action. These suspensions were then diluted and plated on a selective medium containing octopine 10 mM, rifampicin 50 $\mu\text{g ml}^{-1}$ and streptomycin 500 $\mu\text{g ml}^{-1}$. This medium allows only a very limited growth of the recipients which are resistant to the antibiotics but cannot use octopine as a growth substrate. The donors are eliminated by the action of the antibiotics. The transconjugants were selected for by their capacity to utilise octopine and develop into normal colonies. The plates were scored after incubation for 8 d at 25 °C.

* Replacement of arginine plus pyruvate by glucose (2 g l^{-1}) gave almost identical results.

Table 2 Induced and constitutive plasmid transfer

Donor strain	Mating medium	
	Arginine + pyruvate	Octopine
B6S3	$\leq 10^{-7}$	19×10^{-2}
B6S3OCC ^c 6	5×10^{-3}	8×10^{-2}
B6S3OCC ^c 8	$\leq 10^{-7}$	13×10^{-2}

Values are transconjugant frequencies amongst the recipients. Recipient and donor strains were grown in arginine plus pyruvate minimal medium (see Table 1) and the crosses were incubated on arginine plus pyruvate minimal medium or octopine minimal medium. The bacterial mixtures were deposited as drops directly on to the mating medium. The crosses were collected, after incubation for 48 h at 25 °C, with a platinum loop and the bacteria were plated on selective minimal medium (rifampicin 100 µg ml⁻¹, octopine 10 mM) after suspension and dilution in water. After incubation for 4 d at 25 °C the Petri dishes were examined and the colonies scored. The higher efficiency of transfer obtained in these crosses as compared with those in Table 1, indicate that these plate matings result in a more efficient conjugation.

opine found in octopine-containing tumours¹⁶, did not induce conjugative activity.

Experiments performed with some strains carrying nopaline Ti plasmids gave analogous results. Some strains such as K14 and T37 can transfer their plasmid in the presence of 10 mM nopaline. For instance, in a cross between strain K14 as the donor and strain GV3102 (a derivative of strain C58C1 that is resistant to 100 µg ml⁻¹ rifampicin and 100 µg ml⁻¹ erythromycin) performed in conditions analogous to those described in Table 1, some transfer of the Ti plasmid (about 10⁻⁴) was detected in the absence of nopaline, whereas 10⁻² of the recipients had acquired a Ti plasmid when the mating was performed in the presence of nopaline. On the other hand, the low frequency of Ti transfer by another nopaline strain, C58, was not enhanced by adding nopaline to the mating mixture.

As the catabolism of nopaline and octopine is coded for by plasmid genes¹⁷ and is also inducible by the same substrates, it was possible that both the catabolic activity and the transfer activity are under the control of a single regulatory system. One would then expect that both processes would respond to the same inducers, and that some regulatory mutations would be found that would interfere with both of these processes. Five natural opines are now known: octopine, nopaline, octopinic acid, lysopine and histopine. We found that the first four of them can induce both processes (ref. 18) and that the fifth is not an inducer for either process (A.P. and J.T., in preparation). To further explore the situation we performed crosses between strain GV3201 as a recipient and strains B6S3, B6S3OCC^c6 and B6S3OCC^c8, the latter two being spontaneous mutants of B6S3, constitutive for octopine catabolism¹⁸. (Strain GV3201 was derived as a rifampicin resistant mutant from strain B9105 (ref. 19) which was shown to be cured of the two plasmids harboured by the parental B91 strain.) The results shown in Table 2 demonstrate that octopine is required for transfer by B6S3 and B6S3OCC^c8, whereas for B6S3OCC^c6 it took place also in the absence of this inducer.

With the nopaline strain T37, whose Ti plasmid transfer is inducible by nopaline, a mutant, T37NOC^c1, could be isolated that is able to grow on octopine as sole carbon and nitrogen source and was shown to be constitutive for both Ti plasmid transfer and nopaline catabolism¹⁸. The capacity of these constitutive mutants to catabolise octopine seems to be due to the ability of the nopaline-degrading enzyme to use octopine as a substrate as well¹⁸. However, other independently isolated derivatives of the same strain, able to utilise both octopine and nopaline as growth substrate, did not transfer their Ti plasmid in the absence of inducer.

Mutants constitutive for transfer were also selected for

directly, by looking for conjugation without induction: high titre mixtures of donor strain GV3110 (C58C1(TiB6S3)) and recipient strain GV3103 (C58C1 Str^rSp^c) were plated on 0.2% glucose minimal medium. After incubation for 48 h at 28 °C the mixture was inoculated first in a rich medium (0.5% Bacto-beef extract, 0.1% Bacto-yeast extract, 0.5% peptone, 0.5% sucrose in 0.002 M MgSO₄) with 100 µg ml⁻¹ spectinomycin and 100 µg ml⁻¹ streptomycin to eliminate the donor cells and subsequently in octopine minimal medium to enrich for transconjugants. The total transconjugants-recipient population thus obtained was used as donor in a second conjugation without induction with strain GV3102 (C58C1rif^rery^r) as recipient. In this second cross a high level of octopine-utilising transconjugants was obtained. These transconjugants, when used in further crosses, transferred their Ti plasmid at a high frequency (about 10⁻¹) in the absence of octopine and were thus transfer-constitutive.

These results are consistent with the hypothesis of a common regulatory gene controlling two distinct operons, one involved in catabolic activity and the other in plasmid transfer. Our results demonstrate clearly that conjugative activity of the Ti plasmids can be induced by the substrates of some specific enzymes coded for by the Ti plasmids. The types of regulatory mutants that we have been able to obtain thus far are consistent with the idea that a common regulator gene is involved in the control of the activity of two distinct functions—catabolism and transfer.

It is possible that this is but one example of a more general phenomenon. It could be that several bacterial plasmids can be induced to conjugate by a key substance that is a substrate of enzymes coded for by plasmid-borne genes. Some catabolic plasmids and possibly RTF factors might show similar behaviour. Induction of conjugative activity by these conditions that give a selective advantage to plasmid-harboring bacteria would result in efficient spread of the plasmid and thus in a more rapid adaptation of a bacterial population to changes in environmental conditions. Nakazawa and Yokota²⁰ have described a mutant TOL plasmid from *Pseudomonas putida* with increased constitutive levels of metapyrocatechase and increased conjugative activity. The behaviour of this mutant might be explained by the same type of simultaneous regulation of catabolic and transfer functions.

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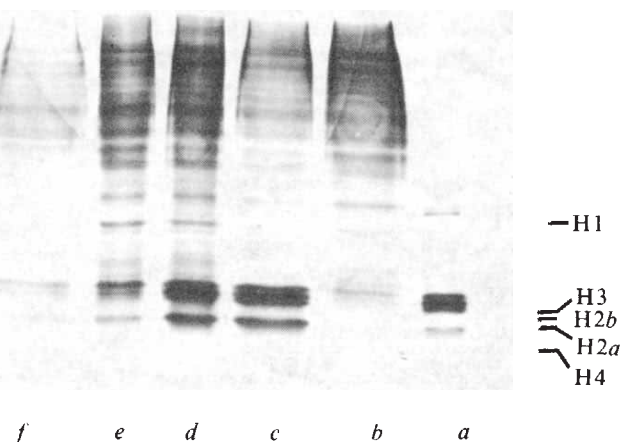
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Functional stabilisation of HeLa cell histone messenger RNAs injected into *Xenopus* oocytes by 3'-OH polyadenylation

WE have shown that, when injected into *Xenopus* oocytes, poly (A) free globin messenger RNA is translated for a short period of time and then rapidly degraded^{1–3}, while, under the same conditions, native mRNA shows a marked stability^{1,2,4}. The poly (A) segment itself is responsible for the stability of native globin mRNA, since its readdition to previously deadenylated mRNA restores the stability of the message⁵. We have shown also that the poly (A) stretch must contain a minimum number of adenylate residues to ensure its protective function⁶. It was interesting to see whether the concept of the stabilisation of eukaryotic mRNAs by 3'-OH polyadenylation is general. This can be done by looking at the effect of the presence of a 3'-OH poly (A) segment on the stability of various mRNAs. Here we study the stability of HeLa cell poly (A)-free histone mRNAs injected into *Xenopus* oocytes, and the effect of 3'-OH polyadenylation on this stability.

An RNA fraction enriched in histone mRNAs (His mRNA) was obtained from polysomes of synchronised HeLa cells in S phase^{7,8}. This RNA preparation, which is not retained on poly (U)-Sepharose on 0.5M KCl at room temperature, directs exclusively the synthesis of human histones in a reticulocyte cell-free protein synthesising system^{7,8}. Fifty oocytes from a single

Fig. 1 Fluorography of a sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis of proteins from oocytes injected with a histone mRNA enriched fraction from HeLa cells (750 $\mu\text{g ml}^{-1}$) (His mRNA). Fifty oocytes from a single *Xenopus laevis* female were injected with the His mRNA preparation. At different times after microinjection, sets of ten oocytes were incubated at 19 °C in Barth medium containing 1 mCi ml^{-1} of ^3H -lysine (67 Ci mmol^{-1}). At the end of the incubations, oocytes were washed and homogenised in Tris-glycine 0.05M pH 8.9. Cell debris and yolk platelets were removed by low speed centrifugation and the supernatant was made 2% in SDS and 5% in mercaptoethanol. Aliquots of the supernatants containing 400,000 dpm of trichloroacetic acid precipitable counts were analysed by electrophoresis on a 15% polyacrylamide slab gel¹⁰. After staining and destaining, gels were treated for and submitted to fluorography¹¹. *a*, Reference ^{14}C HeLa cells histones; *b*, proteins from control oocytes injected with water; *c–f*, proteins from oocytes injected with His mRNA. Incubation periods: *b*, *c*, 0–5 h; *d*, 5–10 h; *e*, 10–20 h; *f*, 20–48 h.



Xenopus laevis female were injected with 50 μl each of a 750 $\mu\text{g ml}^{-1}$ aqueous solution of His mRNA. At different times after injection, sets of ten oocytes were incubated in a saline medium containing 1 mCi ml^{-1} of ^3H -lysine (67 Ci mmol^{-1}) at 19 °C for several hours. At the end of the incubation periods, protein samples were prepared from oocytes and analysed by sodium dodecyl sulphate (SDS)-polyacrylamide slab gel electrophoresis¹⁰. Reference ^{14}C -HeLa cell histones were used as a control. At the end of the electrophoresis, the gels were dried and submitted to fluorography¹¹.

Synthesis of histones H_3 , H_{2b} , H_{2a} and H_4 is clearly detected in oocytes injected with the His mRNA fraction (Fig. 1). The synthesis rate of these four proteins is maximal from 5–10 h after microinjection; it then decreases rapidly and becomes almost undetectable after 20 h (Fig. 1). From this result, we conclude that four naturally poly (A)-lacking mRNAs (or mRNAs possessing a very short poly (A) segment, since they are not retained on poly (U)-Sepharose) behave, as far as their translation is concerned, in a very similar way to globin mRNA from which the poly (A) segment had been artificially removed¹. It thus seems that a 3'-OH poly (A) segment is required to ensure the stability of several eukaryotic mRNAs.

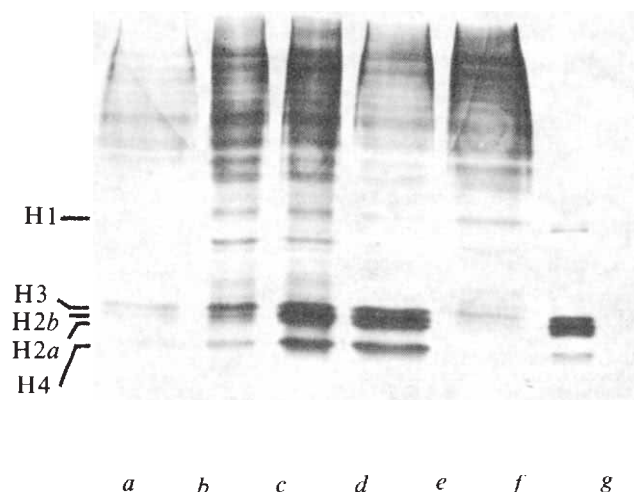


Fig. 2 Fluorography of a SDS-polyacrylamide gel electrophoresis of proteins from oocytes injected with either a native His mRNA fraction from HeLa cells (channels *b–d*) or this His mRNA fraction previously polyadenylated (channels *e–g*). Both RNA preparations have been injected at 750 $\mu\text{g ml}^{-1}$. Eighty oocytes from a single *Xenopus laevis* female were injected either with native His mRNA or with polyadenylated His mRNA (40 oocytes with each preparation). At different times after microinjection, sets of ten oocytes from each batch of cells were incubated at 19 °C in Barth medium containing 1 mCi ml^{-1} of ^3H -lysine (67 Ci mmol^{-1}). At the end of the incubations, oocytes were processed as described in Fig. 1 for gel electrophoresis. *a*, reference ^{14}C HeLa cells histones; oocytes injected with native His mRNA; *e–g*, proteins from oocytes injected with polyadenylated His mRNA. Incubation periods: *b*, *e*, 0–7 h; *c*, *f*, 7–19 h; *d*, *g*, 43–60 h.

To test this assumption, a poly (A) stretch containing 40 to 50 adenylate residues was added to the 3'-OH end of the His mRNA fraction using an ATP : RNA adenylyltransferase from *Escherichia coli*, and the functional stability of this polyadenylated mRNA was tested after microinjection into oocytes. The polyadenylated His mRNA fraction and control poly (A)-free His mRNA were thus injected at a concentration of 750 $\mu\text{g ml}^{-1}$ into two sets of 50 oocytes from the same *Xenopus* female. At different times after microinjection, batches containing 10 oocytes were incubated in a saline medium containing 1 mCi ml^{-1} of ^3H -lysine (67 Ci mmol^{-1}) at 19 °C for several hours. At the end of the incubation periods, protein samples were prepared and analysed by SDS-polyacrylamide slab gel electrophoresis. At the end of the electrophoresis, the gels were dried and submitted to fluorography¹¹.

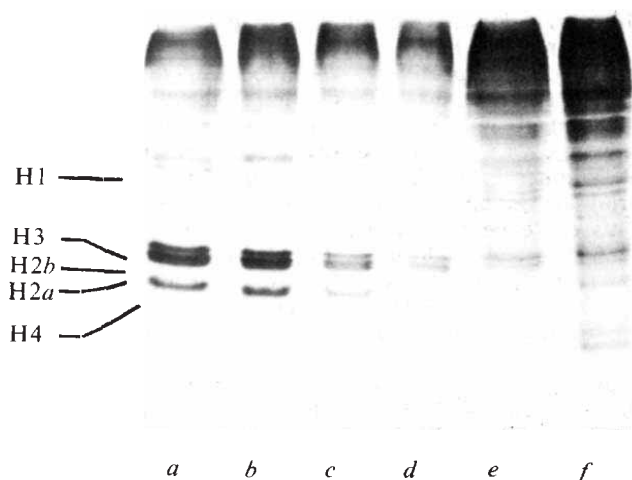


Fig. 3 Fluorography of SDS-polyacrylamide gel electrophoresis of proteins from oocytes injected with native His mRNA ($500 \mu\text{g ml}^{-1}$) supplemented with commercial poly (A) ($500 \mu\text{g ml}^{-1}$). Fifty oocytes from a single *Xenopus laevis* female were injected with native His mRNA supplemented with commercial poly (A). At different times after microinjection, sets of ten oocytes were incubated at 19°C in Barth medium containing 1 mCi ml^{-1} of ^3H lysine (67 Ci mmol^{-1}). At the end of the incubations, oocytes were processed as described at Fig. 1 for gel electrophoresis. *a-d*, proteins from oocytes injected with native His mRNA + commercial poly (A); *e, f*, proteins from control oocytes. Incubation periods; *a, e*, 0–4 h, *b*, 4–20 h, *c*, 20–48 h, *d, f*, 48–60 h.

In complete agreement with the previous experiment, it was found that, in oocytes injected with native His mRNA, the rate of synthesis of H_3 , H_{2a} , H_{2b} and H_4 histones increases during the first 7 h, then decreases during the 7–19 h incubation period and becomes finally hardly detectable 43 h after microinjection (Fig. 2). In the case of polyadenylated His mRNA, however, the synthesis rate of the four histones increases up to 19 h and is still very high more than 43 h after microinjection (Fig. 2). One may thus conclude that the addition of a poly (A) segment at the 3'-OH end of the four naturally poly (A)-free mRNAs coding for H_3 , H_{2a} , H_{2b} and H_4 histones functionally stabilises these messages.

We wanted to see whether poly (A) molecules injected at the same time as His mRNA would have the same effect on the stability of histone mRNAs as terminal polyadenylation. So, we compared, in the same kind of experiment as those described above, the functional stability of His mRNA injected alone (at $500 \mu\text{g ml}^{-1}$) or with commercial poly (A) (100–200 nucleotides long) at $500 \mu\text{g ml}^{-1}$. Figure 3 shows that the presence of poly (A) injected at the same time as the His mRNA fraction does not increase the functional stability of histone mRNAs, although the concentration of poly (A) is approximately ten times higher than when linked to the 3'-OH end of the message molecules, as in the preceding experiment. It thus seems that, in oocytes, the 3'-OH poly (A) segment does not protect the mRNA molecule simply by competitive inhibition of ribonuclease activity as might be inferred by the work of others in *in vitro* experiments¹³. The protection mechanism must be more specific, and we have indeed shown that the degradation of a poly (A)-lacking mRNA is associated with its translation³.

We suggest that the presence of a poly (A) stretch long enough at the 3'-OH end of four different histone mRNAs ensures their functional stability when injected into *Xenopus* oocytes. These results extend our previous conclusion about the stabilising role of poly (A) obtained from experiments using native and deadenylated globin mRNA^{1–3,5,6} (for review see ref. 14). Preliminary results from experiments using sea urchin embryo histone mRNAs confirm our conclusion.

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Role of the G segment in the growth of phage Mu

USING the single-burst technique we have been able to show that close to one-half of the cells in a lysogenic culture of phage Mu yield no viable phage after induction. Our experiments were prompted by an intriguing observation concerning the G segment of Mu, a region of 3,000 base pairs found in either orientation in both mature phages and prophages^{1,2}; it is thought that G inversions are mediated by inverted repeats of approximately 50 base pairs known to be located at the two ends of G (ref. 3). It was observed that progeny phage derived from the induction of lysogenic cultures contain approximately equal numbers of particles with the G segment in either orientation, but virtually all progeny phage derived from lytic cycles of infection have their G segment in one particular orientation⁴.

The simplest proposal to explain this behaviour is based on the following assumptions⁵: (1) inversion of the G segment occurs only in the prophage state. (2) On induction individual lysogenic cells produce bursts in which all phages have the G segment in the same orientation as the parental prophage. (3) Phages with the G segment in one orientation, say G(+), are viable in lytic infection, with the G(–) orientation they are non-viable. (4) The frequency of inversion of G during growth of a Mu lysogen is such that in any culture of 10^8 cells (the minimum required for electron microscope experiments) the G(+)/G(–) ratio is close to 1.

These assumptions lead directly to the prediction that only one-half of the individual cells in a lysogenic Mu culture yield viable phage after induction.

Table 1 Comparison between tubes tested immediately after inoculation

Tubes per set	Tubes containing bacteria (set 1)	Tubes yielding viable phage (set 2)	Expected tubes* from theory yielding viable phage
132	32	20	17
156	62	30	35

The bacterial strain used was a derivative of the *Escherichia coli* K12 strain CSH55 (ref. 10), CSH55Mu⁸/F⁺pro⁺lac::Muets62. To test for the presence of viable phage 0.25 ml of a stationary culture of the indicator bacteria CR63 was added to each tube and these were then incubated overnight at 32 °C. Next day samples from the tubes were spotted onto plates seeded with CR63, and these plates incubated overnight at 37 °C.

*This number makes allowance for the fraction of tubes receiving more than one cell at the multiplicities used.

We set out to test this prediction in experiments reported here, using an adaptation of the classical 'single burst' experiment of Burnet⁶. In the first experiments a culture of a mono-lysogenic bacterial strain containing the heat-inducible prophage Muets62 was grown in broth at 32 °C to 10⁸ cells ml⁻¹. The culture was then diluted appropriately and aliquots distributed amongst two sets of broth-containing tubes with such a multiplicity of bacteria per tube (0.2–0.5) that the majority of tubes which received bacteria were inoculated with a single cell. One set of tubes was then incubated overnight at 32 °C and examined next day to determine the number of tubes containing bacteria; the second set was immediately placed at 43 °C for 60 min (conditions which provoke induction and lysis) and each tube then tested for the presence of viable phage.

Table 1 represents the results of two experiments of this type. The ratio between the numbers of tubes yielding viable phage in one set of tubes and those containing bacteria in the parallel set is close to that predicted by the model. However, a drawback of this kind of experiment is that the numbers involved in the comparison between the two sets of tubes are inevitably small and so possible statistical fluctuations are considerable. To obtain more compelling evidence concerning the basic assumption that the induction of individual lysogenic cells often does not yield viable Mu phage, we modified the experimental procedure to obviate the statistical criticism of the previous results.

The starting point of the modified experiments was similar to the previous ones, but the sets of tubes were now left after inoculation at 32 °C before testing, to allow clones of bacteria to develop in those tubes receiving cells. After various periods of time a set of tubes was removed from 32 °C and the contents of each tube (1 ml) split into two equal volumes. One of these was then assayed to determine the number of cells it contained; the other was induced and then tested for the presence of viable phage.

Table 2 Tests of individual clones for viable phage

Tubes per set	156	
Time of incubation (h)	3	4
No. of tubes containing clones	64	60
Average clone size (no. of bacteria)	28	58
Clones yielding viable phage	46	48
Clones yielding no viable phage	18	12
Fraction of clones yielding no viable phage	0.28	0.20

Bacterial assays were performed by adding 0.5 ml of 1.2% molten Difco agar to each 0.5 ml volume to be tested, shaking the tubes, and then incubating them for 2 d at 30 °C. At this stage spherical colonies could easily be seen and counted. Testing of the other 0.5 ml volumes for viable phage was carried out as in the previous experiment.

Table 2 presents the result of an experiment of this type in which the clones were allowed to develop for 3 or 4 h before testing, at which times they contained an average of 28 and 58 cells respectively. It can be seen that even after 4 h growth there were 12 tubes which, after division, yielded bacteria but no viable phage. The chance that this lack of viable phage was due to the absence of bacteria in the induced volumes is negligible, as in 10 of the 12 tubes the bacterial assays on equivalent volumes were greater than 20. This evidence therefore shows quite clearly that some lysogenic Mu cells yield no viable phage on induction.

Also from Table 2 it can be seen that the fraction of clones yielding no viable phage decreases with increasing time, a result interpreted from the theory as reflecting the occurrence of G inversions during bacterial growth. Using methods similar to those for calculating mutation rates⁷ it is possible to estimate from the data that the rate of G inversions per cell per generation is close to 0.03, and that the G(+)/G(–) ratio in a culture of 10⁸ bacteria derived from a single cell would be within 5% of unity.

Our data therefore confirm the predictions of the G inversion model. They are also consistent with a less restrictive scheme which allows a small amount of inversion to occur during vegetative phage growth. By themselves, however, the data do not prove that our observations are related to the inversion of G. The correlation comes from experiments of a quite different kind performed by Kamp *et al.*⁸. These authors have isolated two types of Mu mutant which are inversion-defective. One has a deletion covering the right end of the G segment, while the other is presumed to be defective in the production of the inversion enzyme. In these mutants, with G locked in its G(–) orientation, mature phage are produced from induced lysogens, but these phage are non-viable in lytic infection.

Taken together these two sets of results provide strong evidence that in a normal lysogenic culture the cells with the G segment of the prophage in the G(–) orientation produce non-viable phage particles. Presumably the G segment contains, at least in part, some gene whose function is essential in the lytic cycle of Mu; and in one orientation this gene is non-functional. There are two possible roles for this gene. It could be involved in a step which must occur early in the lytic cycle of Mu if phage production is to take place, but is not needed when a prophage is induced, or it could affect the structure of the phage coat so that the Mu DNA is not able successfully to penetrate host cells. In both cases non-viable phage with the G(–) orientation would arise from infection and lysogenisation with a G(+) particle followed by a G(+) to G(–) inversion in the prophage state, and finally by induction. The results of Bukhari and Ambrosio⁹ suggest that the second of these alternatives is correct.

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was in the $\downarrow$ (*flip*) orientation⁹. To discover in which way the Mu *flop* particles are defective, we have followed the fate of Mu DNA molecules after infection as well as after induction. In these experiments, the orientation of G in the Mu DNA molecules was determined by cleavage with restriction endonucleases. The principle of such analysis has been enunciated by Allet and Bukhari⁷. We used the enzymes *KpnI* (from *Klebsiella pneumoniae*) and *PstI* (from *Providencia stuartii*) Kahmann *et al.*¹⁰ originally determined the cleavage sites for these enzymes in Mu DNA and found that these enzymes, in combination, are very useful in identifying the G orientation. As shown in Fig. 2, the only *KpnI* cleavage site in Mu DNA is located within G, whereas both *PstI* cuts are outside G, one cut being close to the left of G. Depending on the orientation, a different fragment spanning the left part of G is obtained when Mu DNA is cut with a combination of *KpnI* and *PstI*.

We prepared ³²P-labelled Mu particles, as described by Bukhari and Ljungquist¹¹, and infected *E. coli* cells with these particles. At various times after infection, the cells were centrifuged and the DNA was extracted. The orientation of G in the radioactively labelled Mu DNA molecules was examined by autoradiography after cleavage with *KpnI* and *PstI* and electrophoresis in agarose gels. As shown in Fig. 3, only the *flip* orientation could be detected in the cells. Since the starting

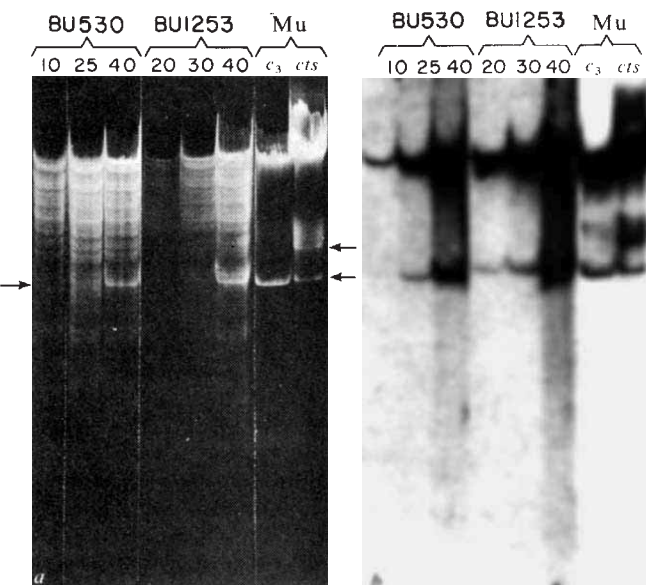


Fig. 4 Orientation of G in replicating Mu DNA after infection. Mu *cts* 62 particles grown by induction were used to infect strain BU530, a prototrophic Mu-sensitive strain of *E. coli* K12, and strain BU1253, an *E. coli* strain carrying the plasmid pRM101, which contains the G segment of phage P1 (see Fig. 3). At various times after infection the cells were washed and the DNA was extracted with phenol. The DNA samples were cut with *KpnI* and *PstI* and fractionated in 1% agarose gels by electrophoresis. The fragments were stained with ethidium bromide and photographed under ultraviolet light. The fragments were then denatured in gels, 'blotted' onto nitrocellulose paper and hybridised with ³²P-labelled Mu DNA^{3,13}. *a*, The ethidium bromide stain photograph is on the left and *b*, the autoradiograph after hybridisation. The right-hand column in both (*a*) and (*b*) shows the Mu *cts* DNA extracted from particles used to infect the cells. This DNA shows both G orientations as compared to Mu *c3* DNA grown by infection, which has only the *flip* orientation. It can be seen in the autoradiograph (*b*), that only the *flip* orientation is detected at different times. That the Mu DNA is replicating is shown by the increase in hybridisation of the Mu fragments. The hybridisation background, particularly visible at 40 min after infection, results from random integration of Mu DNA into the host DNA during growth (see ref. 13). Note that in the ethidium bromide stain at the left the G *flip* fragment is visible at 40 min, indicating a large-scale replication of Mu DNA.

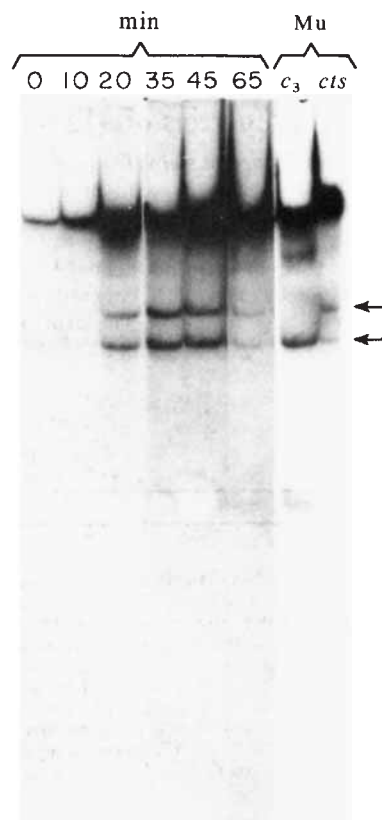


Fig. 5 Orientation of G in replicating Mu DNA after induction. Strain BUHM8305, lysogenic for Mu *cts* 62, was heat-induced by shifting the logarithmically growing culture to 44 °C for 20 min and then shifting to 37 °C. Just before induction and at various times thereafter, samples were withdrawn and DNA was extracted with phenol. The DNA was cut with *KpnI* and *PstI*, fractionated by electrophoresis in 1% agarose gels. The fragments were then denatured and transferred to nitrocellulose paper and hybridised to ³²P-labelled Mu DNA^{3,11}. The figure shows the autoradiograph after hybridisation. The two columns at the right are Mu *cts* (showing both G fragments) and Mu *c3* (showing only the *flip* fragment). Both G orientations are detected in the lysogen before induction. After induction both G fragments increase in amount in parallel. The background hybridisation from 20 min onwards reflects integration of Mu at different sites generating random new fragments that hybridise with Mu DNA¹³. By 65 min the culture has undergone substantial lysis, decreasing the yield of cells and thus the intensity of DNA bands is reduced.

lysate contained both the *flip* and *flop* orientations, this shows that the *flop* particles were not able to infect the cells. Similarly, we found that infected cells contained Mu DNA in the *flip* orientation only whereas the supernatant contained a large population of unadsorbed Mu particles with G in the *flop* orientation. Consistent with this result, we have observed that the Mu phage particles purified after induction of Mu-sensitive host contain proportionally more phage with the *flop* orientation than phage prepared from Mu-resistant host. Presumably, this is because of preferential adsorption of *flip* particles to cell debris during purification.

To determine the orientation of G in the replicating Mu DNA molecules, we either infected *E. coli* cells with unlabelled Mu particles or induced Mu lysogens. The DNA was extracted at various times after infection or induction and cleaved with *KpnI* and *PstI*. The fragments were separated in agarose gels by electrophoresis, denatured and transferred to nitrocellulose paper by the procedure of Southern¹². After hybridisation with ³²P-labelled Mu DNA, the DNA fragments hybridising to Mu DNA (that is, Mu fragments) were visualised by autoradiography, as described previously^{3,13}. Only one orientation of G was detected in replicating Mu DNA, as late as 40 min after

infection (Fig. 4). Even in a strain that contained the G segment of phage μ cloned in a plasmid, and thus had the G inversion system of μ only the *flip* orientation of G could be observed. This shows that the rate of G inversion is slow and that most of the infecting molecules remain in the *flip* configuration throughout the lytic cycle. As shown in Fig. 5, both G orientations were detected in a Mu *cts* lysogen, before or at various times after induction. This result thus provides direct proof that G inversion occurs in lysogenic cells before induction and that, after induction, both orientations replicate equally well. Interestingly, the G orientation of replicating Mu DNA can be determined after infection or after induction, even without DNA-DNA hybridisation and autoradiography. This is because as Mu replicates and Mu DNA copies accumulate, the Mu G fragments stand out above the background. (See Fig. 4.) We have used this method to screen for Mu mutants defective in G inversion (unpublished).

We conclude from these experiments that the reason why Mu particles with only the *flip* (or $\uparrow$) orientation of G are obtained after infection is that the particles with G in the opposite orientation (*flop* or $\downarrow$ orientation) fail to infect the host cells. This block in infection seems to result from an inability of Mu *flop* particles to adsorb to the cells. It is not clear whether the *flop* particles do not adsorb at all or whether the adsorption is reversible such that the *flop* particles are easily detached from the cell surface. Apparently, when lysates are prepared by infection there is a selection for the *flip* particles at each infectious cycle. Since the rate of G inversion is slow, only Mu particles with the *flip* orientation accumulate in the lysates prepared by infection. These results extend the observation of Kamp *et al.*⁹, on the non-viability of the *flop* particles and complement the results of Symonds and Coelho¹⁴ who have shown that only some of the cells in a culture of a Mu lysogen give rise to infectious centres and that the rate of G inversion is slow that is 0.03 per cell per generation.

The inability of the Mu *flop* particles to adsorb properly indicates that these particles lack a phage component needed for adsorption. Evidently, this component is made only when G is in the *flip* orientation. This case provides an interesting example of the flip-flop control of gene expression. A similar mechanism has been postulated for phase variation in *Salmonella*¹⁵.

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Inversion of the G DNA segment of phage Mu controls phage infectivity

THE 3,000 base pair-long G segment of temperate coliphage Mu DNA has been shown to invert spontaneously with high frequency^{1,2}. Inversion, which is independent of the *rec* system of the host³, results in Mu phage with the G segment in either orientation. Nearly equal numbers of both G orientations are observed in a lysogen or when a lysate is obtained by induction of a lysogen. When phage is grown by a series of infections one and the same G orientation prevails⁴. One attractive explanation for these observations implicates inversion of the G segment in the control of phage or host functions. We report here experiments which show that the orientation of the G segment determines infectivity of Mu phage particles and that G inversion is mediated by a phage-specific enzyme system.

In our studies we used a recently isolated plaque-forming substitution mutant, Mu 445-5, which no longer inverts its G segment but apparently has an intact G region⁵. We refer to this phenotype as *gin*⁻ for G inversion. The orientation of the G segment in Mu 445-5 is the same as that found to predominate after lytic infection. We shall call this orientation G(+) and the opposite orientation G(-). To test possible effects of G orientation on Mu, we attempted to invert the G segment of Mu 445-5 *in trans* by using a helper phage. We constructed a double lysogen with Mu 445-5 on the host chromosome and a Mu *gin*⁺ helper inserted in an episome. This double lysogen was induced and the DNA of the phage progeny was analysed by heteroduplex formation with appropriate reference Mu DNA (Fig. 1). The G segment of Mu 445-5 phage DNA produced from the double lysogen was found in either orientation, indicating that the *gin*⁻ phenotype of Mu 445-5 is recessive and can be complemented by a *gin*⁺ phage. This result shows that at least part of the enzyme system responsible for G inversion is specified by Mu itself. Removal of the helper prophage from the double lysogen should result in subsequent dilution of the *gin* enzyme from the cell and thus freeze the G segment of the Mu 445-5 prophage in either the G(+) or the G(-) configuration. Strains that had spontaneously lost the episome carrying the *gin*⁺ helper prophage fell into two classes with approximately equal frequency. The first class released normal plaque forming Mu 445-5 phage, while the second class lysed after heat induction but produced no plaque forming phage. Nevertheless these lysates contained phage particles which could be banded in caesium chloride density gradients and from which normal size DNA could be isolated. The yield and density of these phage particles were virtually the same as for the plaque forming Mu 445-5 phage. Even concentrated lysates did not result in killing or lysogenisation when spotted on a lawn of Mu-sensitive bacteria. As determined by restriction enzyme analysis (Fig. 2), the defective particles obtained from several independently isolated lysogens always contained Mu 445-5 DNA with the G segment in the G(-) configuration, whereas all plaque producing lysogens yielded Mu 445-5 phage with the G segment in the G(+) orientation. The Mu 445-5 *gin*⁻ G(-) lysogens were then re-exposed to the *gin* system provided by the episome with the Mu *gin*⁺ helper prophage. As before, removal of the episome resulted in two classes of lysogens—those producing non-infectious Mu 445-5 G(-) phage and those yielding infectious Mu 445-5 G(+) phage. These results show that the only difference between the defective and non-defective Mu 445-5 phage particles is the orientation of their G segments, and that the inability of the Mu 445-5 *gin*⁻ G(-)

prophage to produce infectious phage can be reversed by inverting its G segment from the (-) to the (+) orientation. Figure 3 illustrates and summarises these experiments.

Because Mu 445-5 has an insertion (which we have shown to be part of insertion sequence IS2) we could not generalise

this conclusion with absolute certainty. However, indirect evidence suggests that Mu wild-type phage with a G(-) configuration is also defective. It was found that not all cells lysogenic for a temperature-inducible Mu produce phage after heat induction (D. K., unpublished). In view of the

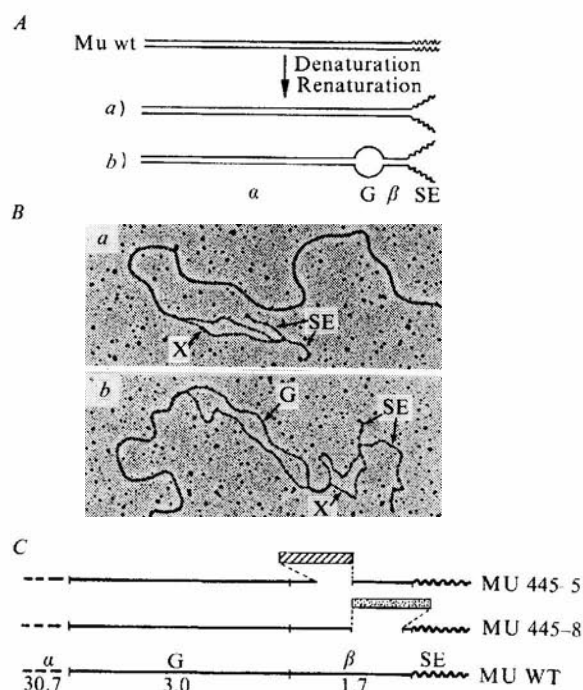


Fig. 1 Determination of the G segment orientation by heteroduplex analysis. *A*, Schematic presentation of DNA structures observed after denaturation and self-annealing of wild type Mu DNA extracted from phage grown by induction⁴. The various segments are designated as alpha, G, beta, and split ends (SE). The lengths of the heterogenous bacterial DNA comprising the split ends (~) are variable and range from a few hundred to about 3,000 nucleotides. In about half the heteroduplexes (*a*), the G segment is duplex. The single-stranded G loop in the other half (*b*), is due to annealing of Mu strands with G segments in opposite orientations, a result of inversion. *B*, EM heteroduplex determination of the orientations of the G segment in Mu 445-5 grown in the presence of a helper phage. DNA species were denatured with 0.2 M NaOH, neutralised with 2 M Tris-HCl, pH 7.2, and then reannealed at room temperature for 50 min in 50% formamide at a total DNA concentration of 4 μ g ml⁻¹. Renaturation was stopped by dialysis against 0.01 M Tris, 0.001 M EDTA, pH 8.5 at 4°C. Sample grids for electron microscopy were prepared by the formamide variation of the Kleinschmidt technique¹¹⁻¹³. Micrographs were taken with a Philips 201C electron microscope and printed at about the same magnification. Only the right ends of the heteroduplexes are shown. DNA was isolated from phage produced after heat induction of the double lysogen DK850 which is Δ pro lac Sm^R (Mu cIts62 445-5)/F' pro lacI::Mu cIts62 Lts (the Mu Lts phage was obtained from A. I. Bukhari¹⁴). The orientation of the G segment of the Mu 445-5 phage was determined from heteroduplexes formed with Mu 445-8. Mu 445-8 is a deletion-substitution mutant in the right half of beta⁵ and was propagated by infection so that its G segment was in the (+) orientation. Mu 445-5 is a deletion substitution mutant in the left half of beta⁵. Heteroduplexes between Mu 445-5 and Mu 445-8 can be distinguished from those of Mu 445-5/Mu or Mu/Mu 445-8 because of their non-overlapping substitution-deletions (see *C*). *B-a*, A heteroduplex of Mu cIts62 445-5/Mu 445-8, showing a duplex G region, a split end and the noncomplementary substitution loop (X) in beta. The G segment of Mu cIts62 445-5 is in the G(+) orientation. *B-b*, A heteroduplex of Mu cIts62 445-5/Mu 445-8 showing the noncomplementary loop in the beta region identical to (*a*), a split end and a G loop, indicating that the G segment of the Mu cIts62 445-5 phage is present in the G(-) configuration. *C*, Diagrammatic map of the G beta region of phages Mu 445-5 and Mu 445-8. Solid lines represent Mu DNA. Deletions are indicated by gaps and substitutions by shaded bars. Sizes of the individual segments are given in kilobase pairs.

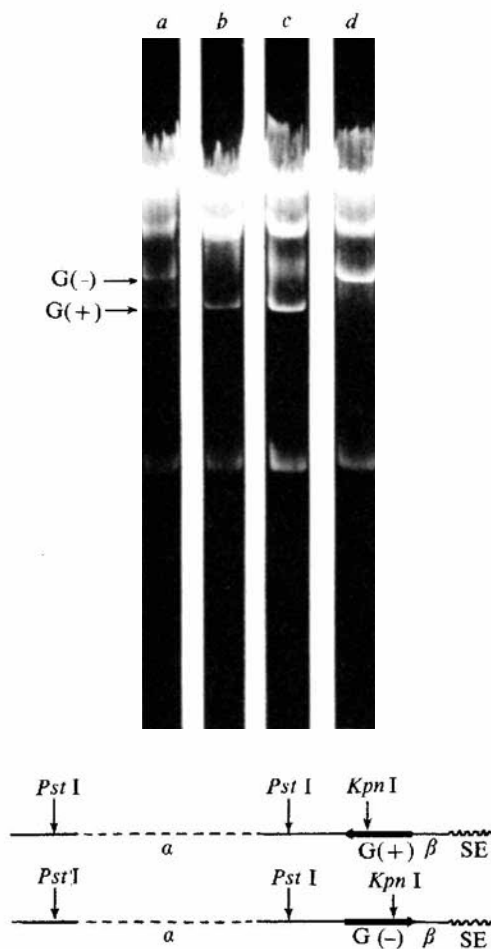


Fig. 2 Determination of the G segment orientation by agarose gel electrophoresis of Mu DNA cleaved with restriction enzymes. The principle of analysing the G segment orientation with restriction enzymes, first described by Allet and Bukhari who used the enzyme *Hind*III⁷, is based on the cleavage of Mu DNA with an enzyme that cleaves the G segment at least at one asymmetrically located site, such that DNA containing G in the (+) orientation produces a restriction fragment of one size while DNA with G in the (-) orientation yields a fragment of different size. Except for Mu *vir*, which was grown by infection, all other phages were propagated by induction. Phage and DNA preparation have been described previously¹⁵. Samples of 2 μ g DNA were digested at 37°C with an appropriate amount of *Pst*I and *Kpn*I restriction endonucleases in a buffer containing 0.06 M Tris-HCl (pH 7.9), 0.06 M MgCl₂, 0.06 M 2-mercaptoethanol. After digestion, sucrose and bromophenol blue were added to final concentrations of 5% and 0.02% respectively. Slab gel electrophoresis was through 1.2% agarose for 4 h under a constant voltage of 120 V, essentially as described by Sugden *et al.*¹⁶. After staining with ethidium bromide, bands were visualised by excitation with short wave ultraviolet light and photographed through a Kodak no. 23 red filter with Polaroid film P/N55. The *Pst*I and *Kpn*I cleavage maps of Mu DNA¹⁷ are shown below. *Pst*I-*Kpn*I fragments of different size are obtained in digests of Mu cIts62 DNA (*a*), because of the presence of both G segment orientations. The smaller of these fragments is characteristic of the orientation found in Mu *vir* (*b*) and in Mu cIts62 445-5 G(+) (*c*) DNA and is indicated by G(+). The larger fragment is produced when the G segment is in the G(-) configuration, as in Mu cIts62 445-5 G(-) DNA (*d*). It co-migrates with the diffuse right end fragment. The largest band represents the *Pst*I fragment generated from the alpha region of the Mu genome. The second largest band represents the *Pst*I fragment from the right end, which remained uncleaved by *Kpn*I due to low enzyme activity. The smallest fragment originates from the left end of the molecule.

results with Mu 445-, it was tempting to speculate that this phenomenon, found independently by Symonds and Coelho (personal communication), reflects the existence of a sub-population of Mu wild-type lysogens that produces only defective G(-) phage particles on induction. The Mu 445-5 *gin*⁻ lysogens provided the necessary control to test this assertion, because a population of these lysogens is homogeneous with respect to the orientation of the G segment. In fact, every Mu 445-5 G(+) lysogen gives rise to an infectious centre after heat induction. In contrast, for every *gin*⁺ strain tested thus far the frequency of infectious centres may vary from experiment to experiment but never reaches 100% (Fig. 4). Furthermore, this variation seems to reflect the proportion of the prophages which are in the G(+) orientation, because analysis of Mu *gin*⁺ DNAs shows that the ratio of G(+) to G(-) phage also varies. When phage lysates were prepared from the same cultures that had been tested for infectious centres, and DNA from these phages was analysed for the orientation of G segments, we found that the percentage of phage chromosomes with a G(+) orientation was directly proportional to the yield of infectious centres of each culture (Fig. 4). If one assumes that the G segment hardly ever rotates in an induced lysogen, most lysogens would produce either G(+) or G(-) phage. Because there is no significant difference in the yield of G(+) and G(-) phage particles, the ratio of G(+) to G(-) phage in the lysate would then equal the ratio of G(+) to G(-) prophages at the time of heat induction. Thus the yield of infectious centres can be correlated with the ratio of G(+) to G(-) prophages. This implies that Mu *gin*⁺ prophages, which are temporarily in a G(-) configuration when heat induced, produce defective phage particles.

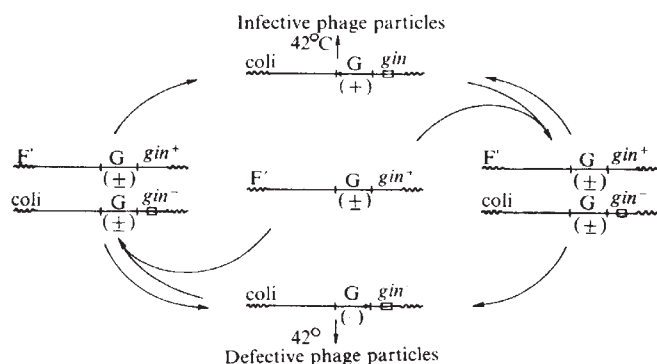


Fig. 3 Inversion of the G segment of Mu 445-5 promoted by a Mu helper phage. DK984: Δlac pro Sm^R (Mu cIts62 445-5 G(+) *gin*⁻) was used as a recipient for mating with DK654: Δlac pro Sm^R/F' pro *lacI*:: Mu cIts62 Lts G(+) *gin*⁺. Growth and mating conditions were described by Miller¹⁸. Transconjugants were selected as red colonies (*lac*⁺) on MacConkey lactose streptomycin plates. The double lysogens were grown overnight before they were streaked out on MacConkey lactose plates. Colonies that had been cured of the episome spontaneously occurred with a frequency of about 0.1-1% and were identified as white colonies (*lac*⁻). After restreaking, these colonies were tested for Mu phage production by stabbing them into a lawn of Mu-sensitive indicator bacteria. Thirty to fifty colonies were tested in each step. Three cycles of conversion of G(+) to G(-) and back to G(+) were conducted without any apparent change in the outcome of the experiment. To exclude the possibility that G segments are exchanged by recombination between the episome and chromosome rather than that they are inverted intramolecularly, two control experiments were performed. Rotation of the G segment, G(+)→G(-)→G(+), was not affected by a *recA* mutation of the host. Second, an episome carrying a Mu 445-5 *gin*⁻ G(+) prophage instead of a Mu *gin*⁺ helper was introduced into a strain lysogenic for Mu 445-5 *gin*⁻ G(-) and this double lysogen was subjected to the procedure as described above. Out of 36 colonies that had been cured of the episome, none had acquired a Mu 445-5 *gin*⁻ G(+) prophage.

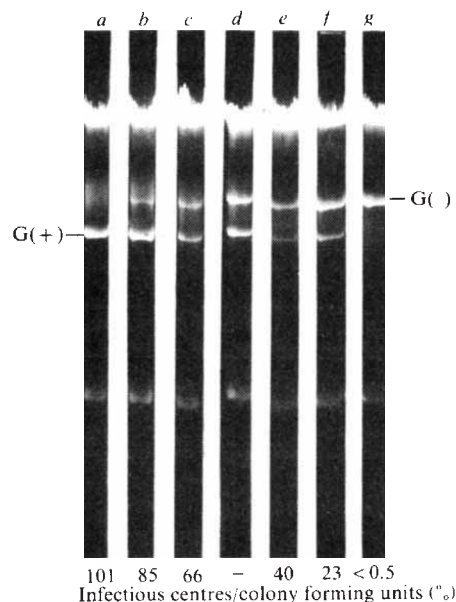


Fig. 4 Infectious centre production of various strains lysogenic for Mu and its correlation to the orientation of the G segment. Analysis of phage DNAs with restriction enzymes *PstI* and *KpnI* was as described in Fig. 2. Samples of the lysogenic cultures were taken immediately before heat induction and were diluted in broth to about 1×10^3 colony forming units ml⁻¹. One aliquot was plated at 34 °C to measure the number of cells. In order to determine the number of infectious centres, another aliquot was heat induced at 42 °C for 10 min, mixed with Mu-sensitive indicator bacteria and plated with soft agar at 40 °C. The ratio of infectious centres to colony forming units is given in % of average numbers derived from 7-10 parallel platings. In every experiment, 50-100 colonies from plates used to determine the number of colony forming units were routinely tested for lysogeny and all were found to produce plaque forming phage. This excludes trivial explanations for the lower yield of infectious centres such as frequent spontaneous loss of the prophage or frequent mutation resulting in a defective phage. The results for the following Mu lysogens are shown: a, DK948: (Mu cIts62 445-5 G(+)); b, DK588: (Mu cIts62 *mom*⁻); c, DK392: (Mu cIts62 *cII*31); d, DK1018: (Mu cIts62 445-5 G(-))/F' pro *lacI*:: Mu cIts62 445-5 G(+); e, DK394: (Mu cIts62); f, DK722: (Mu cIts62 *mom*⁻ Mu); g, DK947: (Mu cIts62 445-5 G(-)). The double lysogen in (d) is included as a standard for a 50:50 ratio of G(+) and G(-) molecules. The bacterial hosts are CSH50: Δlac pro Sm^R in (a), (d), (g) and the prototrophic K12 strain W8 in (b), (c), (e) and (f). DK722 was derived from DK588 by selecting for resistance against Mu *vir*. The *mom*⁺ and the *cII*¹⁹ mutations have been described elsewhere. The absolute numbers for infectious centres and the G(+) / G(-) ratios are not reproducible for a given Mu lysogen unless it is *gin*⁻, and the numbers differ from culture to culture within the range that is shown here. Only the correlation between % infectious centres and G(+) / G(-) is consistently found to be the same. The different genetic make-up of the lysogens therefore does not account for the difference in absolute values.

This finding is consistent with our results obtained in *gin*⁻ conditions.

These results have to be reconciled with the current view that all essential functions of phage Mu are located in the alpha region to the left of the G segment⁶. The *mom* function, which is possibly controlled by G inversion, is not essential for Mu growth⁸. Our preliminary findings that Mu 445-5 G(-) phage is deficient for the S gene product raise the possibility that expression of the S function depends on the orientation of the G segment. The S gene, which has been mapped to the immediate left of the G segment³, could in fact be located partially in the G segment and then be split by G inversion. A recent refinement of the correlation between the physical and genetic map at this particular location (M. Howe, personal communication) seems to support this view. An alternative explanation

would be that a promoter is coupled to the S gene only when the G segment is in the (+) orientation. This kind of mechanism has been proposed in the case of *Salmonella* phase variation⁹. Genome rearrangement has also been envisaged as a means of controlling gene expression in eukaryotic cells¹⁰. Therefore the *gin* mediated inversion of the G segment in phage Mu is a useful paradigm for hypothetical control mechanisms that might play a part in the expression of genes in other prokaryotic as well as eukaryotic organisms.

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Involvement of phage Mu-1 early functions in Mu-mediated chromosomal rearrangements

THE temperate phage Mu-1 not only integrates at any location in the chromosome of its host *Escherichia coli*¹ but also provokes different types of chromosomal aberrations: insertions of extrachromosomal circular DNA²⁻⁴, translocations, deletions and inversions of chromosomal segments (refs 5, 6, Howe and Bukhari, unpublished results and M.F., in preparation). Immunity to phage Mu prevents the induction of such events, indicating that Mu gene expression is required for these rearrangements. Furthermore, the direct participation of Mu DNA in the generation of chromosomal aberrations is demonstrated by the observations that one entire Mu genome is always adjacent to the site of the deletions, two entire Mu genomes in the same orientation flank the inserted and translocated DNA fragments, and inverted chromosomal segments are surrounded by two entire Mu genomes in opposite orientations (ref. 22 and M.F., in preparation). Dependence of such Mu-induced alteration on the A and B early phage functions was investigated. A

mutants of Mu are unable to mediate all the events tested, while B⁻ mutants still promote deletion. Based on the results presented here, and on other data, we propose that the A function directly concerns the insertion of the phage and that the B gene codes for a replication function.

The molecular mechanism by which Mu causes chromosomal rearrangements is not known, although all observations made so far are consistent with the idea that the Mu enzymes and the sites on Mu DNA used for normal Mu integration are necessary and sufficient for Mu to mediate these events⁸. All mutants of Mu which result in altered capacity to integrate have been found to affect two of the early functions, either A or B. Such mutants neither replicate nor synthesise late Mu-specific mRNA^{9,10} and on infection of a sensitive host they are unable to mediate integration of extrachromosomal DNA^{2,3}. When present as an induced prophage, A⁻ mutants still show that defect, but B⁻ mutants do efficiently mediate integration events⁴. We have studied the capacity of A⁻ and B⁻ prophages to mediate deletions and translocation events.

Mu-mediated deletion formation can be readily detected in strains which carry a Mucts62 prophage in the *trp* operon (*trp* : : Mucts62). After growth at 37 °C, leading to partial induction of the thermoinducible prophage, clones with a deletion in the *tonB trp* region (for revised map of *E. coli*, see ref. 11) may be selected (at 30 °C) for their resistance to phage Φ80 and the col VB colicins. The frequency at which *tonB* deletions appear in partially induced Mu lysogens is ~10⁻⁴, that is ~500 times higher than the spontaneous frequency of 10⁻⁶-10⁻⁷ at which deletions are formed in that particular region (see Table 1). *sup*⁺ and *sup*⁻ strains were isolated which carry either a Mucts62 *Aam* or a Mucts62 *Bam* or a Mucts62 X (Mucts62 B⁻ : IS1 (ref. 12)) prophage in the *trp* operon. These were induced at 37 °C (42 °C in the case of Mu X lysogen) and the frequencies at which *tonB-trp* deletions were generated were measured. The frequency of appearance of *tonB* clones

Table 1. Induction of *tonB* deletions by induced Mucts62 A⁻ and Mucts62 B⁻ prophages inserted in the *trp* operon

Induced strain		Frequency of appearance of <i>tonB</i> clones
<i>supF</i> ⁻ <i>trp</i> : : (Mucts62 <i>Aam</i> 1093)	Positive control	2.6 × 10 ⁻⁵
<i>supF</i> ⁻ <i>trp</i> : : (Mucts62 <i>Bam</i> 1066)		4 × 10 ⁻⁵
<i>sup</i> ⁺ <i>trp</i> : : (Mucts62)		10 ⁻⁴
<i>sup</i> ⁺ <i>trp</i> : : (Mucts62 <i>Bam</i> 1066)		1.1 × 10 ⁻⁴
<i>trp</i> : : (Mucts62 X3600)		2 × 10 ^{-4*}
<i>sup</i> ⁺ <i>trp</i> : : (Mucts62 <i>Aam</i> 1093)		1.8 × 10 ⁻⁶
<i>sup</i> ⁺ (Muc ⁺) <i>trp</i> : : (Mucts62)	Negative control	1.2 × 10 ⁻⁶
<i>supF</i> ⁻ <i>trp</i> ⁺		2 × 10 ⁻⁷

sup⁺ strains are derived from CSH54 (ref. 17) and are Δ*pro-lac*, *his*⁻, *thi*⁻ str^R. *supF*⁻ strains are derived from QD5003 (ref. 18) HfrH *supF*⁻. The *trp* : : (Mucts62 X 3600) is derived from HC-B-7-18 Hfr P4X *metB*, *argEC-1*. Muam mutations were described by Howe¹⁸. Cultures were grown overnight at 37 °C (42 °C) in L broth, starting from a single colony, without aeration. One loopfull of each culture was resuspended in 5 ml L broth and incubated up to stationary phase without aeration at 37 °C (42 °C). Cultures were titrated on LB plates at 30 °C. 0.1 ml of the 10⁻¹ dilutions was mixed with 0.2 ml of Φ80vir (10¹¹ phage ml⁻¹) and 0.2 ml of a col VB lysate, incubated for 20 min at 30 °C and titered on LB plates, seeded with 0.1 ml of the col VB lysate. The frequency of appearance of *tonB* clones was calculated from the ratio no. of clones on LB + col VB / no. clones on LB.

* Experiment performed at 42 °C: 10 *tonB* clones derived from the Mucts62 X lysogen were analysed. They all retained the immunity and S genes of the Mu prophage, showing that none of the deletions extended into the Mu prophage. This was done to check that none of the deletions was mediated by the IS1 present in the B gene of the Mucts62 X rather than by Mu itself.

Table 2 Translocation of the *asd* gene on to an *Flacpro* episome by Mucts62 *Aam1093* and Mucts62 *Bam1066* prophages inserted in *malA*

Donor	Frequency of <i>asd</i> ⁺ translocation
(Mucts62 <i>Aam1093</i>) <i>sup</i> ⁺ / <i>Flacpro</i>	4×10^{-8}
(Mucts62 <i>Aam1093</i>) <i>supF</i> ⁻ / <i>Flacpro</i>	3×10^{-6}
(Mucts62 <i>Bam1066</i>) <i>sup</i> ⁺ / <i>Flacpro</i>	2×10^{-8}
(Mucts62 <i>Bam1066</i>) <i>supF</i> ⁻ / <i>Flacpro</i>	5×10^{-6}

The strains were grown in LB broth at 30 °C to $\sim 2 \times 10^8$ ml⁻¹; 0.5 ml of both the donor *Flacpro/malA* :: (Mucts62*am*) *recA sup*⁺ or *supF*⁻ and the recipient *argG, metA, asd, thi, lac, recA, Rif*^R (Muc⁺), were mated for 2 h at 42 °C. *Asd*⁺ sexductants were selected by plating dilutions of the mating mixture on minimal medium supplemented with glucose, arginine, methionine and rifampicin. The frequency of *asd* transfer which is taken as the measure of Mu-mediated translocation, is given by the ratio no. of *asd* sexductants/input of F' cells.

in strains used as positive control, that is, the *supF*⁻ *trp* : : (Mucts62 *Aam*) and *supF*⁻ *trp* : : (Mucts62 *Bam*) in which the A and B mutations were suppressed, was $\sim 10^{-4}$. The same frequency was found with *sup*⁺ strains lysogenic for either Mu*Bam* or Mu*X* mutants where the B gene was not expressed. However, in the *sup*⁺ strain lysogenic for Mu *Aam*, *tonB* clones appeared at a frequency of 10^{-6} , this being the same frequency as that found in the strain used as negative control (*trp* : : (Mucts62) (Muc⁺)) in which no thermal induction of Mu occurred.

This clearly shows that the A gene product is required for Mu-mediated deletion formation, while the B gene product is dispensable. Mu-mediated translocation occurs both after infection with Mu of a sensitive bacterium and in induced Mu lysogens. This can be readily detected in an F' strain, by transferring the episome to a recipient in which it is possible to select for plasmids which have acquired a given translocated marker. When the prophage is fully induced proximal and distal host markers are translocated at the same frequency⁷. We tested the ability of A⁻ and B⁻ induced Mu prophages to mediate translocation of proximal markers. We isolated *sup*⁺/F' *pro lac* strains, carrying either a Mucts62 *Aam* or a Mucts62 *Bam* prophage in the *malA* gene, and their *sup*⁻ derivative which were used as positive controls. These strains were mated at 42 °C with a recipient suitable for selection of transferred F' having eventually acquired and *asd* gene (which is located near *malA* (ref. 11)). The results in Table 2 show that translocation of the *asd*⁺ allele occurs at a frequency of 3.5×10^{-6} in the *sup*⁻ lysogens, while in the *sup*⁺ lysogens this frequency is $\sim 4 \times 10^{-8}$, thus ~ 100 times lower. Translocation by an induced Mucts62 *X* prophage was also tested (data not

shown) and was found to be negative. From these results we conclude that both the A and B gene products are required for Mu-mediated translocation.

As an alternative approach, prophages affected in their ability to mediate translocation, were isolated after treatment of a Mucts62 lysogen with nitrosoguanidine. The strain used contains an F' *lac* episome, and a Mucts62 prophage in the *trp* operon. After mutagenesis we screened for clones which after induction neither produced viable phage nor transferred episomes with translocated markers. We shall ignore mutants which were found to directly affect the transfer of the F' episome (4% of the total number of clones screened). Ten mutants (1.3% of the clones tested) in which the deficiency was found to be in the Mu prophage were analysed for their ability to mediate deletion of the *tonB* chromosomal marker and to translocate markers proximal to the prophage, on to the F *lac* episome. They were also tested for their capacity to complement A⁻, B⁻ and L⁻ mutants of Mu. All mutants fall into two classes (see Table 3): some (2/10) complement A⁻ and L⁻, but not B⁻ mutants of Mu, could mediate formation of *tonB* deletions and could not mediate translocation; the others (8/10) complemented the L⁻ mutant; some complemented B⁻ mutants but none complemented A⁻ mutants, nor could any mediate either deletion formation or translocation of chromosomal markers. This confirmed the findings that the B gene product is not required for deletion formation while the A gene product is necessary, and that both A and B products are essential for Mu to mediated translocation. Mu-mediated deletions and insertions are events which can occur in an induced Mu lysogen at the site where the prophage is originally located and therefore do not necessarily involve transposition of Mu DNA. On the other hand, Mu-mediated translocation always involves transposition of the Mu genome. On the basis of our previous work⁸, we conclude that two Mu copies are always involved in Mu-mediated illegitimate recombination. As long as the Mu prophage is integrated into the host chromosome which is the case in the induced lysogen since no extensive excision of the Mu genome occurs after induction¹³, the two copies of Mu can be generated through the replication of the host genome. However, as soon as Mu leaves the host chromosome, most probably as a heterogeneous circle containing host DNA covalently linked to one Mu copy¹⁴⁻¹⁶ it must be able to replicate by itself to generate two copies. One would thus expect Mu replication function(s) to be dispensable for those chromosomal rearrangements which can occur at the site of the prophage, that is, deletions and insertions, but these

Table 3 Properties of the prophage mutants selected for their inability to transpose host markers

Mutant isolated	Frequency of translocation on <i>Flac</i> of		Frequency of <i>tonB</i> deletion	Complementation of mu mutants in gene		
	<i>pyrF</i> ⁺	<i>thyA</i> ⁺		A	B	L
1	2×10^{-7}	3.4×10^{-6}	5.3×10^{-6}	—	+	+
2	1.6×10^{-6}	9×10^{-6}	5×10^{-5}	+	—	+
3	1.5×10^{-7}	6×10^{-7}	2.3×10^{-6}	—	+	+
4	1.6×10^{-7}	10^{-6}	1.1×10^{-4}	+	—	+
5	8×10^{-8}	1.6×10^{-7}	1.7×10^{-6}	—	+	+
6	2.4×10^{-7}	1.6×10^{-6}	4.7×10^{-6}	—	—	+
7	4.7×10^{-7}	2.9×10^{-6}	2.2×10^{-6}	—	—	+
8	8×10^{-8}	2.5×10^{-6}	6.5×10^{-6}	—	—	+
9	2.4×10^{-7}	4×10^{-6}	5.2×10^{-6}	—	—	+
10	1.2×10^{-7}	2×10^{-6}	4.5×10^{-6}	—	+	+
Mucts62	2.3×10^{-5}	8×10^{-5}	1.5×10^{-4}	+	+	+

MuA⁻ is Mucts62 *Aam1093* (ref. 19). MuB⁻ is mucts62 *Bam1066* (ref. 19). MuL⁻ is Mucts62 *Lts5017* (ref. 20). The strain from which the mutants were derived contained a Mucts62 prophage in the *trp* operon and an *Flac* episome. The method for measuring the *tonB* deletion frequency is described in Table 1. The frequency of translocation of the *pyrF*⁺ or *thyA*⁺ alleles on to the *Flac* were determined as described in Table 2 using a *pyrF*⁻, *thyA*⁻, (Muc⁺), Spc^R *recA*⁻ recipient. Complementation tests were performed by introducing F' *lac* Mucts62*am* episome in F⁻ *recA*⁻ derivative of the mutated lysogens and measuring phage production on *sup*⁺ and *sup*⁻ indicator bacteria, after induction of the dilysogens.

functions should be essential for translocations which always involve transposition of Mu DNA and duplication of the phage genome outside of the host chromosome. This is the case for the B gene product, and therefore we propose that the B gene product is involved in Mu replication. Mu integration function(s) are, on the contrary, expected to be required for all Mu-mediated chromosomal rearrangements. Thus, as only A^- mutants of Mu are unable to mediate deletion formation, integration of extrachromosomal DNA and translocation, we propose that the A gene product is an integration function. The same conclusion has been reached independently by O'Day *et al.*²¹: they found that only MuA^- mutants are completely defective for integration.

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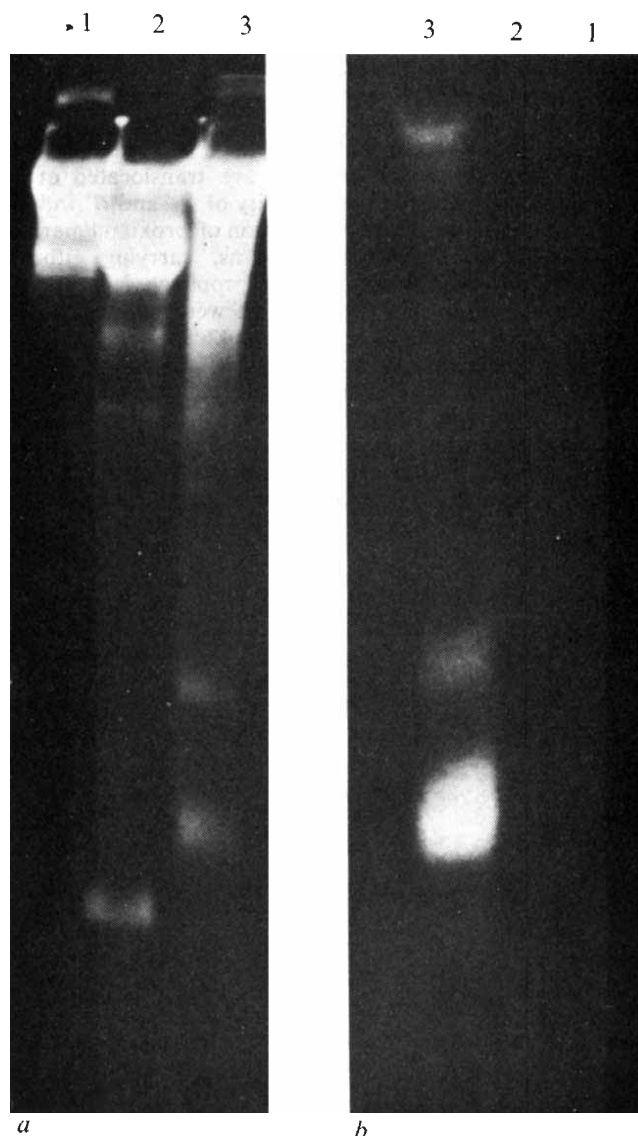
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Immunoglobulin light-chain structural gene sequences cloned in a bacterial plasmid

IMMUNOGLOBULIN light-chain mRNA molecules and their cDNA transcripts have served as molecular probes in hybridisation experiments designed to quantify the number of immunoglobulin genes¹⁻¹³ and to characterise the arrangement of these genes in the mammalian genome^{14,15}. In other studies these cRNAs and cDNAs have been used as substrates for nucleotide sequence analyses to define the structural gene encoding

immunoglobulin light chains^{16,17}. Unfortunately, two central technical problems have developed during these studies. First, the immunoglobulin mRNA cannot be obtained in amounts sufficient for many experiments. For example, determination of the complete nucleotide sequence of this RNA remains difficult without more material. Second, immunoglobulin mRNA cannot be obtained as a homogeneous RNA completely free of other RNA species. These impurities have probably interfered with several hybridisation studies. In order to obtain a substrate for nucleotide sequence analysis as well as to provide a pure hybridisation probe we have cloned the DNA (cDNA) complementary to the immunoglobulin light-chain (kappa) mRNA from the mouse myeloma MOPC-149. The

Fig. 1 DNAs from plasmids (1) pCR1, (2) pBG2 (ref. 20), and (3) pCR1- κ 40 were digested with S1 nuclease in 40% formamide and fractionated on a 2% agarose gel run from top to bottom. The exact conditions for digestion of the DNA are as described by Hofstetter *et al.*²⁵. *a*, Bands identified by ethidium bromide staining. *b*, An autoradiogram obtained after the DNA from the lanes shown in (*a*) was denatured and transferred to a nitrocellulose filter, and hybridised to ³²P-MOPC-149 cDNA as described by Southern²⁷. The size of the pCR1- κ 40 insert fragment was determined to be 700 base pairs by calibration with *Hae*III fragments of ColEI DNA (marker fragments not shown). The pBG2 insert fragment (*a*, lane 2) is 576±50 base pairs (ref. 15).



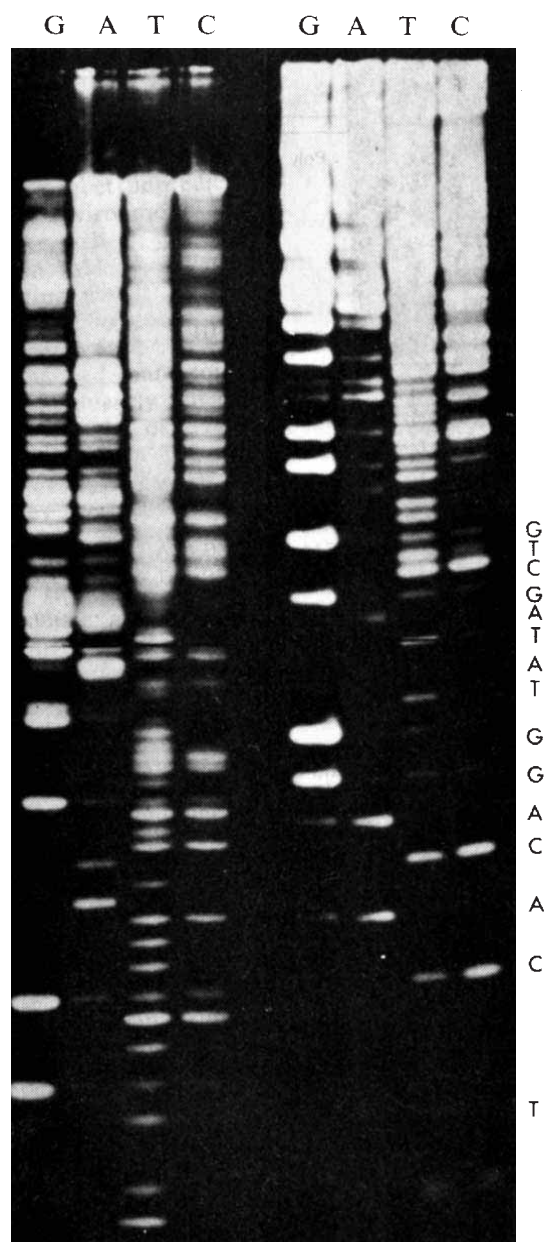


Fig. 2 Radioautograph for determination of the nucleotide sequence starting from the *Hae*III site in *pCRI-κ40* reaching towards the *Pst*I site. The 850-base pairs *Hae*III fragment found in digests of *pCRI-κ40* was isolated from an acrylamide gel, kinased, and cleaved with *Pst*I (see Fig. 3 for location of restriction sites). The 325-base pair fragment generated in this way was then subjected to the reactions described by Maxam and Gilbert³⁰. G, A, T, C across the top of the radioautograph indicate the specificity of the reaction. Two sets of reactions were electrophoresed for 19 h (a) and 8 h (b). The letters on the right side of the figure indicate the nucleotide sequence suggested by the band pattern. A few of the deduced residues are indicated as examples. These nucleotides correspond to residues 3-17 from the *Hae*III cleavage site. The complete sequence deduced from these autoradiograms is shown in Fig. 3.

plasmid *pCRI-κ40* is shown here to contain sequences corresponding to 700 bases of the immunoglobulin mRNA.

The cDNAs derived from several polyadenylated mRNAs have been converted into double-stranded DNA and joined to plasmid vectors using the poly dA-T tailing procedure¹⁸ or the synthetic oligonucleotide linker method¹⁹. The insertion of rabbit β-haemoglobin²⁰ and rat insulin¹⁹ mRNA sequences into plasmids has been confirmed by nucleotide sequence

analysis of the cloned DNA segment. Other insert carrying plasmids have been shown to hybridise to MOPC-173 immunoglobulin light-chain cDNA²¹, mouse α and β haemoglobin cDNA²², and ovalbumin cDNA²³, suggesting that the sequences of these mRNAs have also been cloned in plasmids. We used a slight modification of the poly dA-T tailing procedure, first used for cloning the rabbit β haemoglobin cDNA^{18,20} sequences, to clone immunoglobulin cDNA and have unambiguously confirmed its presence in the clone by determining a portion of its nucleotide sequence.

We began with the total poly A-containing mRNA found on the polysomes of the mouse myeloma tumour MOPC-149 rather than pure immunoglobulin mRNA. (The purest fractions of 14S mRNA were reserved for the screening procedures used to identify clones containing immunoglobulin sequences.) Immunoglobulin light-chain mRNA is only 10-30% of this RNA population. The cDNA obtained by reverse transcription of this population of mRNAs was found to be very heterogeneous in size, 50-2,000 bases in length (J.G.S., unpublished). Nevertheless, we used this heterogeneous cDNA as a substrate for *Escherichia coli* DNA polymerase I to generate double-stranded DNA hairpin structures. These hairpin structures were converted into double-stranded DNA by digestion with S1 nuclease. Then, poly dA stretches were attached to the double-stranded DNA, and poly dT stretches were added to linear plasmid *pCRI* DNA (which had been cleaved with *Eco*R1) by the action of terminal nucleotidyl transferase (kindly provided by R. L. Ratliff) using the procedure described by Wu *et al.*²⁴. Finally, the poly dA 'tails' on the double-stranded DNA were annealed to the poly dT 'tails' of the plasmid DNA to form circular hybrid molecules.

In this way a large number of hybrid molecules were formed. To amplify and purify a hybrid molecule containing immunoglobulin gene sequences we used these hybrids to transform *E. coli*. The NIH Advisory Committee on Recombinant DNA has decided that this type of experiment requires an EK2 host-vector system and a P3 level of physical containment. We complied with these requirements. We therefore used the crippled EK2 host, *E. coli* strain κ1776, with the approved bacterial plasmid *pCRI*. When 0.8 μg of hybrid molecules were used to transform *E. coli* strain κ1776 we obtained 112 independent transformed clones (that is, cells which had received the kanamycin resistant phenotype conferred by *pCRI*).

As expected, not all of these 112 clones contained immunoglobulin sequences. These 112 clones were grown on nitrocellulose filters, and hybridised to highly purified ¹²⁵I-labelled

Table 1 Extent of hybridisation of MOPC-149 cDNA to various plasmid DNAs

Unlabelled sequences	MOPC-149 ³ H-cDNA S1-nuclease resistant c.p.m. (%)
<i>pCRI-κ40</i>	135 (66)
<i>pCRI-λ52</i>	21 (10)
<i>pCRI</i>	32 (16)
MOPC-149 mRNA	209 (100)

Full-length, purified ³H-MOPC-149 cDNA was hybridised to an excess of unlabelled plasmid DNA, and the amount of hybrid formed was measured as the amount of S1-nuclease resistant material formed during hybridisation²⁵. The plasmid *pCRI-λ52* was constructed in the same fashion as plasmid *pCRI-κ40*, except that total poly A-containing mRNA from the myeloma tumour RPC-20 was used to make the initial cDNA transcripts. 200 c.p.m. of MOPC-149 cDNA was added to 0.5 μg sonicated plasmid DNA or 20 ng of MOPC-149 mRNA, in 0.6 M NaCl, 50 mM Tris pH 7.5, in a final reaction volume of 50 μl. The samples were boiled, placed at 70 °C and allowed to hybridise for 2 h. Finally, the samples were diluted into 1 ml of S1-nuclease buffer (0.04 M NaCl, 0.03 M sodium acetate pH 4.5, 0.12 M ZnSO₄) divided into two equal portions and one portion was digested with 5 μl S1-nuclease for 1 h at 45 °C. The number of trichloroacetic acid-precipitable counts remaining after S1 digestion was taken as a measure of the amount of hybrid formed.

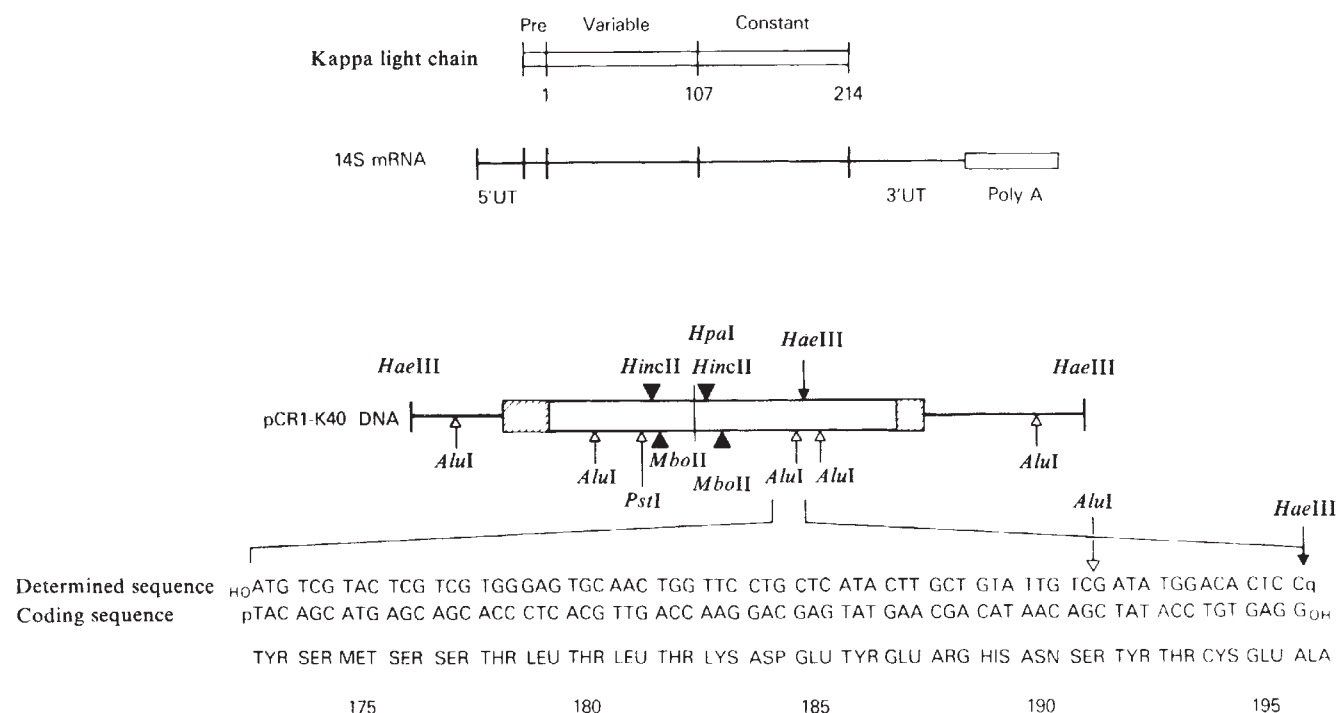


Fig. 3 The arrangement of the immunoglobulin light-chain gene sequences inserted into pCR1- κ 40 and the location of restriction enzyme sites in the inserted sequence. Kappa light chain represents a single polypeptide (amino terminus left; carboxyl terminus right) formed during *in vitro* translation of 14S immunoglobulin mRNA. The polypeptide has been divided by vertical lines into three regions, a 20-amino acid putative precursor sequence, a 107-amino acid variable region sequence and a 107-amino acid constant region sequence. The numbers below the vertical lines indicate the amino acids at the ends of the variable and constant regions. 14S immunoglobulin light-chain mRNA, drawn with its 5' end at the left, is roughly 1,200 bases long (including about 200 bases of poly A at the 3' end). The regions directly below the variable and constant regions of the light chain represent the two 321-base sequences required to encode these portions of the polypeptide. 5' UT and 3' UT are the parts of the mRNA which are not translated into protein, and are found near the 5' and 3' ends of the mRNA. The pCR1- κ 40 DNA segment shown includes the insert sequence, as well as the plasmid sequences (represented by a straight line) and the poly dA-T linkers (cross-hatching) that join the insert to the plasmid. The determined nucleotide sequence (see Fig. 2) was found to be from the anti-sense strand. The coding sequence, deduced from the anti-sense sequence, was found to correspond to amino acids 173-196 of the constant region (bottom row). Most of the restriction enzyme sites, (*Pst*I, one *Hinc*II/*Hpa*I, one *Mbo*II, and one *Alu*I site) were determined by digesting the 325-base pair fragment or the 525-base pair fragment, obtained by *Pst*I cleavage of the kinase-treated 850-base pair *Hae*III fragment, with each enzyme. Similar multiple enzyme digests using kinase-labelled fragments were used to identify the other indicated cleavage sites. *Mbo*II, and *Alu*I each cleave the insert sequence in at least one more location, but these locations have not been defined. We have noted that the deduced sequence contains a *Hinc*II recognition sequence (at the sequence encoding amino acid 178/179), however while some evidence for partial cleavage at this site has been found, the enzyme prefers the other two sites at least threefold.

14S mRNA obtained from MOPC-149 myeloma tumours using the procedure of Grunstein and Hogness²¹. Sixteen of the 112 clones hybridised to purified mRNA (data not shown). The DNAs from these 16 clones were then screened for their ability to protect highly purified MOPC-149 cDNA from S1-nuclease digestion. For example, the DNA from pCR1- κ 40 was able to protect 66% of a MOPC-149 cDNA from S1-nuclease digestion (Table 1). pCR1- κ 40 DNA protected more MOPC-149 cDNA than the DNAs from any of the other 15 clones, so this clone was chosen for further analysis.

One problem with the poly dA-T joiner method is that insertion of a sequence at the restriction endonuclease *Eco*R1 cleavage site within the plasmid DNA destroys the *Eco*R1 site. Thus the insert sequence cannot be cleanly excised from the plasmid DNA by the action of a single restriction enzyme. To overcome this problem Weissman and co-workers²⁵ have developed a technique which cuts the poly dA-T joiner sequences and excises the insert sequence as a single fragment. As poly dA-T has a lower melting temperature than any other sequence, these sequences can be preferentially denatured in 40% formamide. These sequences are then susceptible to S1-nuclease digestion. The fragment excised in this way can be separated from the rest of the plasmid DNA by gel electrophoresis. The results of such an experiment performed on pCR1- κ 40 DNA are shown in Fig. 1a. Two fragments are excised from

pCR1- κ 40 DNA, but not from pCR1 DNA. Both of these fragments specifically hybridise to MOPC-149 cDNA (Fig. 1b). We suggest that the fainter, slower band is a partial digestion product, reflecting the presence of an easily denatured sequence in the plasmid pCR1. We believe that the faster-moving fragment, present in high yield, contains the insert sequence free of any plasmid sequences. The size of the inserted sequence seems to be 700 ± 50 base pairs. Consistent with this result was the finding that the R loop²⁶ formed between pCR1- κ 40 DNA and MOPC-149 mRNA was about 700 base pairs long (J.G.S., M. Sullivan, and B. Peterlin, unpublished).

The sequence inserted into pCR1- κ 40 is only 700 ± 50 bases long whereas immunoglobulin mRNA (not including the poly A) is 950 bases long, indicating that about 200 bases of light-chain mRNA sequences must be missing from the cloned sequence. Which portions of the mRNA are not included in the inserted sequence in pCR1- κ 40? To answer this question, and in order unambiguously to identify the cloned segment, we identified the restriction endonuclease cleavage sites in the inserted sequence, determined the nucleotide sequences around these cleavage sites, and then correlated the nucleotide sequences with the amino acid sequence which they encode.

We have found six restriction endonucleases that cleave the inserted sequence in pCR1- κ 40. pCR1 and pCR1- κ 40 DNA were digested with many restriction enzymes and electro-

phoresed on polyacrylamide or agarose gels; enzymes with cleavage sites in the insert sequence showed at least one more band in the pCR1- κ 40 digests than in the pCR1 digest. These results were confirmed by blotting the fragments onto nitrocellulose paper and hybridising to 32 P-labelled MOPC-149 cDNA as described by Southern²⁷. If an enzyme cleaved the insert sequence then label would hybridise to two bands rather than just a single band. In this way the restriction enzymes *Mbo*II, *Alu*I, *Hae*III, *Hpa*I, *Hinc*II, and *Pst*I were each shown to cleave the inserted sequence at least once, while the enzymes *Bam*HI, *Hind*III, *Hha*I, *Sst*I, *Hinf*I, *Hae*II, *Hpa*II, *Kpn*I and *Bgl*II do not seem to cleave the inserted sequence.

The nucleotide sequence of a small portion of the inserted sequence in pCR1- κ 40 was determined in order to demonstrate conclusively that the inserted sequence could encode for a portion of light-chain amino acid sequence. An 850-base pair fragment from a *Hae*III digest of pCR1- κ 40 DNA was isolated, labelled at its 5' ends with 32 P, cleaved into two pieces (a 525-base pair fragment and a 325-base pair fragment) with *Pst*I (see Fig. 3), and finally the 325-base pair fragment was subjected to the standard reactions for sequencing DNA fragments of Maxam and Gilbert³⁰ (Fig. 2). The nucleotide sequence determined from this procedure is shown in Fig. 3. This nucleotide sequence when read in the correct frame correlates perfectly with a 23-amino acid sequence from the immunoglobulin kappa-type constant region, and with the RNA sequence predicted by Milstein *et al.*²⁸. Thus, we conclude that an immunoglobulin sequence has been inserted into pCR1- κ 40. Using this nucleotide sequence to orientate the map we were able to determine the arrangement of immunoglobulin sequences in pCR1- κ 40 (Fig. 3). Further, the plasmid pCR1- κ 40 contains about 700 base pairs of the immunoglobulin gene sequence, spanning a portion of the 3' untranslated region, the entire constant region and over 150 base pairs of MOPC-149 variable region gene sequence.

This hybrid molecule will be of great value for nucleotide sequence analysis, identification and cloning of genomic DNA segments containing actual immunoglobulin genes and the unambiguous determination of their arrangement in chromosomal DNA. Indeed, we have already used pCR1- κ 40 to obtain two interesting chromosomal DNA fragments containing immunoglobulin variable region sequences (J.G.S., M.H.E., D. Tiemeier, S. Tilghman, F. Polsky, A. Leder, P.L., unpublished).

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Corrigendum

In the letter 'Detection of casein messenger RNA in hormone-dependent mammary cancer by molecular hybridisation' by J. M. Rosen & S. H. Socher, *Nature* **269**, 83–86, the legend to Fig. 4 should start 'Measurement of casein mRNA in hormone-independent mouse mammary tumours. . . .' (not hormone-dependent). Similarly, line 2 on page 86 should read '. . . in the hormone-independent mouse mammary carcinomas . . .'.

Errata

In the letter 'Birefringence signals and calcium transients in skeletal muscle' by G. Suarez-Kurtz & I. Parker, *Nature* **270**, page 748, the horizontal calibration in Fig. 3a should read 50 ms, not 10 ms.

In the letter 'Circular structures of large scale and great age on the Earth's surface' by J. M. Saul, *Nature* **271**, 345–349, Figs 1 and 2 were reproduced to the wrong scale so that the transposition marks do not correspond. The scale reduction of 1:1,000,000 in Fig. 1 legend is also incorrect. The correct versions of these figures can be obtained in reprints of the letter.

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matters arising

Biostratigraphy of Seymour Island, Antarctica

THE paper by Hall on Cretaceous and Tertiary dinoflagellates from Seymour Island, Antarctica¹ contains misleading information. In particular the stratigraphic columns in Fig. 1 do not represent the known field relations. The overlap of sections S-13 and S-3 seems most unlikely on geological grounds; the sections are less than 3 km apart, the sediments have different provenances, and represent different facies (S-13 is delta plain, and the lower part of S-3 is prodelta or delta slope, and palaeo-current indicators suggest southeastward flow). The base of the measured section S-3 is not the base of that part of the sequence. Section S-11 occurs below the unconformity above which S-13 is located; the Palaeocene age may well be correct, but there is no overlap in time with S-13. The age of the beds at Cape Wiman (S-16) is open to debate, but the correlation indicated in Fig. 1 is an oversimplification of the possible interpretations.

The ammonite-dated strata form part of a homoclinal sequence dipping to the south-east and occupying the whole of the southern half of Seymour Island. Howarth² figured several ammonites from near the base of the section, and one ammonite from near where the unidentified ammonite was found that contains 'early Senonian' dinoflagellates in the matrix. Howarth assigned an Early to Middle Campanian, or possible late Campanian age, to the ammonites he described. The strata from which the 'unidentified' ammonite comes are unconsolidated, so that fossils are found, with few exceptions as loose material on dip slope surfaces or at the base of short sections. The ammonite was collected on a dip slope. The southern half of Seymour Island, unlike the northern part north of Cross Valley, lacks glacial debris, and therefore the ammonite cannot be considered a glacial erratic. The 'unidentified ammonite' belongs to the genus *Maorites*, is identified specifically as *M. densicostatus* (Kilian and Reboul), and others of this species are figured by Howarth². Hall's postulate of an 'early Senonian' age seems to be in conflict with the ammonite data. Until proven otherwise, the most likely explanation of this apparent conflict is that the dinoflagellates had been

reworked from older strata or, as stated by Sarjeant³, that the exact ranges of these dinoflagellate species are uncertain.

Finally we would note that Simpson⁴ regards the so-called Miocene beds as Late Eocene, though possibly either Middle Eocene or Early Oligocene. Furthermore, Von Ihering⁵ recognised the Eocene affinities of the molluscs.

Hall's data are very valuable, but some of the statements and conclusions are misleading for those not familiar with the local geology. Papers on the stratigraphy and palaeontology of the Tertiary rocks on Seymour Island have been presented at the Third Symposium on Antarctic Geology and Geophysics at Madison, Wisconsin, in August, 1977.

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HALL REPLIES—In 1975 I examined sediment samples from four measured sections on Seymour Island, Antarctic Peninsula in a pilot study of dinoflagellates, spores, and pollen. A series of palynologic assemblages from two sections, S-3 and S-11, contained numerous diagnostic dinoflagellates; the sections were provisionally assigned a late Eocene-early Oligocene age and a Palaeocene age, respectively. However, sections S-13 and S-16, the correlations of which Elliot and Zinsmeister are critical, are represented collectively only by three samples. Concerning age dating, I reported that "neither of the samples from S-13 provides conclusive information". I also suggested a Palaeocene or late Cretaceous age for S-16 based on one dinoflagellate assemblage. Not only did I report the unsureness of the correlations of S-13 and S-16, but question marks were drafted adjacent these sections in Fig. 1 of ref. 1, further stressing the uncertainty of the correlation of these two sparsely sampled sections. The correlations shown in the figure that are misleading to Elliot and Zinsmeister are clarified on reading the text of the article.

The dinoflagellate species *Cyclonephelium distinctum* and *Deflandrea cretacea* have greater ranges than I acknowledged in the article. Thus my

age assignment of 'early Senonian' to the ammonite matrix (unidentified when I submitted the manuscript but subsequently referred to *Maorites densicostatus*) is indeed unwarranted.

Elliot and Zinsmeister cite Simpson's² review of fossil penguin material and Von Ihering's³ 1927 molluscan studies from Seymour Island as previous interpretations of an Eocene age for these beds, an implied criticism that my corresponding conclusion based on dinoflagellates is less than original. I give Simpson full credit for his conclusions in my article: "The dinoflagellates evidence supports Simpson's conclusion that the fossil penguin materials from Seymour Island, presumably from the upper part of section S-3 or its equivalent, are no older than late Eocene and no younger than early Oligocene". Von Ihering's fortuitous interpretation in 1927 of an Eocene age for the Seymour Island Series was disputed by Antarctic scholars for over 40 yr; the beds were regarded as Miocene in age⁴ until Simpson's vertebrate work and the supporting evidence of the dinoflagellates.

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Asymmetrical displacement currents

KOSTYUK *et al.*¹ have recorded an asymmetrical displacement current in snail neurones (*Helix pomatia*) which, because of a similar voltage dependence, they have associated with the calcium conductance change caused by membrane depolarization. It was noted that the characteristics of the 'calcium gating currents' in snail¹ and *Aplysia*² neurones were different, and it was suggested that at least part of the difference was due to a technical problem with series resistance. However, we think that there are indeed actual differences in the displacement currents recorded in the two neurones.

First, in voltage-clamp studies in snail neurones, it has not been possible to demonstrate separate channels with

different kinetics or voltage dependence for sodium and calcium ions³⁻⁵. Consequently the displacement current recorded in snail neurones¹, although sharing the voltage dependence of calcium ionic current, may be associated with gating of sodium channels, gating of calcium channels, or gating of common channels through which both sodium and calcium ions can pass. In contrast, separate, distinguishable channels for sodium and calcium ions have been demonstrated clearly in *Aplysia* neurones because of differences in kinetics and voltage dependence^{2,6}. Thus a displacement current with properties (particularly voltage dependence) similar to those of the calcium conductance change but distinct from those of the sodium conductance change can reasonably be called a calcium gating current. If the displacement current recorded in snail neurones¹ is not gating channels selective for calcium ions, as seems possible, it could well be different from the calcium gating current in *Aplysia* neurones.

Second, we believe that the slow rising phase of the calcium gating current recorded in *Aplysia* at 6°C is real and not due to slow establishment of a new clamp potential, as suggested by Kostyuk *et al.*¹, for the following reasons. (1) As we have stated², new clamp potentials were reached within 100 μ s. (2) The measured series resistance was always less than 0.5% of the membrane resistance. We have calculated that the time constant of the change in membrane potential across the surface membrane would be less than 100 μ s. (3) The rising phase of the calcium gating current is preceded by an 'immediate on, exponential off' asymmetrical displacement current which we believe is sodium gating current for the reasons given². If the slow rising phase of the following calcium gating current were due to slow establishment of the clamp potential, the time course and voltage dependence of the earlier displacement current would be difficult to explain. (4) The time to peak of the calcium gating current has a high Q_{10} of about 3. This temperature sensitivity can hardly be due to slow establishment of new potential levels across the surface membrane. (5) The time to peak of the calcium gating current can be varied by changing the holding potential while keeping the clamp step amplitude constant, again difficult to explain in terms of slow clamping.

We must conclude therefore that the slow rise of calcium gating current we have recorded in *Aplysia* neurones² is real and cannot be explained as suggested by Kostyuk *et al.*¹ If the displacement current recorded by

Kostyuk *et al.*¹ is indeed a true calcium gating current, there may be real differences in calcium gating currents in the surface membranes of the two nerve cells.

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KOSTYUK *et al.* REPLY—Adams and Gage have suggested several additional arguments in favour of the existence of a rising phase in the development of Ca gating current recorded in the neurone of *Aplysia*. But they do not consider the problem of membrane potential settling time in the two microelectrode voltage clamp experiments. They say that the "control of membrane potential . . . was achieved in less than 100 μ sec"¹. But the rapid changes of potential measured at the microelectrode input can strongly differ from the real (and much slower) changes in the membrane potential. This difficulty in microelectrode voltage clamp experiments has been mentioned by Geduldig and Gruener² in their investigation of *Aplysia* neurones. It is due to the existence of a capacitance between closely situated microelectrodes used to measure potential and inject feedback current. Detailed analysis of the corresponding circuit is quite complicated (some useful information can be found in ref. 3). The settling time of a new clamp potential can be estimated from the termination of current capacitive transient only. The membrane current curves demonstrated in Fig. 1 by Adams and Gage¹ (although presented on a contracted time scale) suggest that the capacitive transient in these experiments lasted for several ms. The time-to-peak of the asymmetrical current presented in Fig. 3 is in qualitative agreement with that length.

These arguments are not intended to question the relationship of the recorded asymmetrical current¹ to the movement of gating charges. But it seems likely that the rate of decay of the 'early' component and the delayed rising phase of the 'late' component reflect the conditions of the experiment rather than the real kinetics of gating currents. Other effects suggested as

arguments in favour of the 'natural' origin of the rising phase can also be explained in another way. Thus the cooling of the preparation results in an increase in membrane resistance. It is equivalent to an increase in the internal resistance of the current source which charges the membrane capacitance.

The discovery of a true rising phase in the gating current would be very important for the description of gating charge movement⁴. But the experimental problems of sufficiently fast membrane potential settling are difficult to solve and must be treated cautiously⁵. As to the separation of specific Na and Ca channels in molluscan neurones, this was achieved recently with the help of the intracellular dialysis technique⁶.

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Multispecific antibodies

VARIOUS points were raised by Secher¹ in a comment on our recent letter on "Evidence for multispecificity of antibody molecules²."

One question concerned the biological significance of cross reactions having binding constants of about 10^5 l mol⁻¹. We showed that these cross reactions will fix complement and lyse red cells²; one can conclude that if they can induce cytolysis, they are likely to be of biological significance. On the other hand, the concentration of antibody (and target antigen) has an important role. If one assumes a titre of 150 μ g ml⁻¹ of IgG (that is, 10^{-6} M) and, for simplicity, a similar one for the antigen (although if it is on a cell membrane one really cannot make an estimate), then, for a binding constant of 10^5 l mol⁻¹, about 10% of the antibody will be in the bound form. This is a conservative estimate since it has been shown^{3,4} that the 'functional' affinity is larger by a factor of 10^3 to 10^4 than the association constant for the binding of a monovalent hapten. The binding interaction would be even more favoured if the antigen were concentrated in a particular area, for example, bacterial antigens in a local infection, or, as in the case of putative autoimmune diseases, antigenic structures on tissues and organs.

Secher calls our analogy between antibodies and enzymes "not very appropriate" because "enzymes are homogeneous and so multispecificity in substrate binding could not enhance their specificity . . .". Perhaps we were unclear in our paper. We were not trying to draw a functional analogy but were trying to indicate that all protein-ligand interactions rely on the same play of short range forces and geometry. Hence, if enzymes are multispecific, it should not be surprising to find that antibodies are also multispecific.

We should like to comment also on Secher's suggestion that the best antibodies (with binding constants of 10^9 – 10^7 l mol⁻¹) may be highly specific (that is, not multispecific). From our point of view, this is not a logical conclusion. Whether a binding constant for a ligand is 10^9 l mol⁻¹ or 10^6 l mol⁻¹, the character of the binding site of the antibody is no different. It still should be able to accommodate other ligands and interact with them. Presumably the binding constant of a cross reaction could be even higher than that of the reaction with the immunogen.

We should like to thank Secher for reminding us of the early suggestions of Talmage⁵. We are embarrassed by our failure to cite his paper.

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SECHER REPLIES - Enzymes (and antibodies) can bind ligands (antigens) other than the 'primary ligand'. Ligands with very similar chemical structure may bind almost as tightly as the main ligand and one might expect to find a series of compounds of decreasing association constant and decreasing chemical similarity to the primary ligand. The concept of multispecificity (as used by Inman for example) is different. It implies that two or more antigens of unrelated structure can bind specifically to a single antibody molecule. This could take place at distinct sites, overlapping sites, or the same site, but presumably will use different contacts between the antibody and the antigen.

The former phenomenon is well known for both enzymes and antibodies. However there are only a few claims of examples of multispecificity

(in both systems), and even these are controversial.

With reference to the specific points that Cameron and Erlanger raise above. Binding of antigen is only one aspect of the biological significance of a cross-reaction. The ability to trigger lymphocytes into division and antibody production must also be considered, and also the extent to which such cross-reactions are representative of the total antibody population or are rare exceptions.

I agree that if "enzymes are multispecific then it should not be surprising to find that antibodies are also multispecific". To what extent these generalities have been demonstrated is still not clear (see above for definition of multispecificity).

The final sentence of my original article was intended to be taken as pure speculation and not as a logical conclusion.

Cross-reactions with binding (association) constant higher than that of the immunogen do indeed exist and have been named 'heteroclitic'.

(An error in my original article has been corrected²).

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Marsupial trophoblast and mammalian evolution

WE present here a view of marsupial-eutherian origins and evolutionary trends, which constitutes an alternative to the view of Lillegraven^{1,2} recently discussed by Cox in a *Nature News and Views* article³.

The trophoblast constitutes the outer foetal boundary of the eutherian chorioallantoic placenta where it functions as the major foetal component of the placental barrier as well as a site of endocrine activity. The trophoblast is also a foetal component of the eutherian yolk sac placenta. In most marsupials, maternal-foetal transfers occur solely through a yolk sac placenta where trophoblast associates with both vascular and avascular regions. An important exception occurs in the marsupial bandicoots where both yolk sac and chorioallantoic placenta develop; trophoblast differs in form regionally within the boundaries of each type of placenta⁴⁻⁶.

The trophoblastic layer persists until term in the chorioallantoic placenta of all mammals except bandicoots, in which the layer disappears shortly before term⁴⁻⁶. Lillegraven, however, states (page 713, ref. 2) that "tropho-

blast is strictly found only in placental mammals," thus misquoting his authorities, Davies and Hesseldahl, who say that trophoblast is a peculiar mammalian structure. Lillegraven (page 720, ref. 2) states further that "The 'invention' of trophoblastic tissues by primaevial eutherians was probably the single most important evolutionary event in the history of the infraclass". This has led to the mistaken conclusion that it is the evolution of trophoblast which enables only Eutheria to obviate immunological crisis during pregnancy^{2,3}.

Lillegraven says (page 101, ref. 1) that "... true implantation by erosion of the maternal epithelium never occurs in marsupials". It has long been known that in the bandicoot chorioallantoic placenta trophoblast disappears as a layer late in gestation⁴⁻⁶, probably through fusion with the uterine luminal epithelium⁶. Comparable fusion may also occur during early implantation of certain Eutheria⁴. Similar, less extensive invasion of uterine epithelium by trophoblastic cells occurs at the yolk sac placenta in several marsupials (such as in *Dasyurus viverrinus* and *Sminthopsis crassicaudata*), the uterine epithelial layer becoming modified at the placental site by interaction of foetal and maternal tissues during implantation.

Thus, evidence concerning trophoblast and uterine erosion tends to unite rather than separate evolutionary aspects of eutherian and marsupial placentation. Although it is not known whether marsupial trophoblast contains immunological or endocrine properties comparable with those proposed for eutherian trophoblast⁷, attention is being focused on this important problem⁸. Because trophoblast is certainly not a eutherian innovation, the argument for the "competitive inferiority" of marsupials is considerably weakened, as Kirsch⁹, on other grounds, has also concluded.

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reviews

Bridging the two cultures

Eric Ashby

A Sense of the Future. By J. Bronowski. Pp. 286. (Massachusetts Institute of Technology Press: Boston, Massachusetts and London, 1977.) \$12.50; £7.25.

JACOB BRONOWSKI was one of those rare people who deserve the title of "Renaissance Man". Academics are suspicious of Renaissance Men. A mathematician who can write perceptively about Blake, compose a play, publish poetry which isn't doggerel, and do research ranging from the history of ideas to the technology of coal: such a man must surely be "unsound". But for this prejudice among pedants, Bronowski would surely have been taken more seriously than in fact he was. No doubt there are better coal technologists, better poets, better literary critics, better philosophers, than Bronowski was. The value of his contribution to contemporary thought rests upon his capacity to see the relationships between these disciplines, to create patterns in which the disciplines are intertwined. This is what he did in *Science and Human Values* and in *The Identity of Man*. (It is significant that both these books are based on lectures he gave in the USA: Americans are more hospitable to Renaissance Men than the British are.) And his television series, *The Ascent of Man*, was a heroic attempt to achieve his ambition: "to create a philosophy for the twentieth century which shall be all of one piece". He drew his viewers into participation over questions which have perplexed intellectuals for a millennium.

Anyone who writes and talks as fluently as Bronowski did is saturated with invitations to contribute articles and to give lectures. Characteristically, Bronowski generously accepted many of these invitations and took immense pains to make his contributions elegant and polished. In *A Sense of the Future*, nineteen of these, covering the years 1952 to 1974, have been selected and edited for publication. They comprise his reflections on science. Another volume is promised, which will contain his reflections on aesthetics and literature.

The themes of these essays are familiar to those who have read Bronowski's books. He generates a

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stream of traffic across the bridge between the Two Cultures (a bridge which, oddly enough, C. P. Snow seems never to have discovered). In science, he says, the experiment conceived in imagination is tested by confronting it with physical experience; in literature, the imaginative conception is tested by confronting it with human experience. Experiments and conceptions which survive become part of the cultural heritage. In science, as in humanism, excellence depends on the quality of imagination. In the pursuit of science Bronowski sees also the recipe for ethics; for to pursue science is to search for truth, and no one can verify for himself all the truths he needs to know. Therefore, he has to trust the word of other people. Therefore, the principle of truthfulness "binds society together, because without it the individual would be helpless to tell the truth from the false"; it is a cement to hold society together. So his "social axiom" is that we ought to act in such a way that what is true can be verified to be so.

The most thoughtful essays in the book are about evolution. Bronowski expounds with admirable clarity the

difference between the 'closed' teleology of (say) a watch, whose purpose is implicit in the way its parts are put together, and the 'open' teleology of evolution, where there is selection for 'fitness for change' to meet unpredictable changes in the environment. Of evolution, Bronowski writes, we can say "what Piet Hein said of a work of art, in a penetrating phrase: that it solves a problem which we could not formulate until it was solved".

It is commonly assumed that people who write and lecture fluently possess an inexhaustible reservoir of fresh ideas. This assumption, even for the Bronowskis of this world, is incorrect. Therefore, when they are pressed with invitations to talk here and to write there, they either have to appear churlish, and refuse, or they have to cannibalise some of their own earlier work. So I'm not surprised that there is some repetition in these essays, between chapters 7 and 8, 9 and 10, 12 and 13, 15 and 16. If Bronowski himself had edited these essays, I guess he would have chosen one or the other, not both, of these overlapping pairs. His editors may be criticised for not having done this. I am grateful for the repetitions. They reveal how unsolved problems lodged in Bronowski's mind for years, to be taken out and re-examined from time to time. It is instructive to watch him reflecting on the same subtle idea (for instance, the *Entscheidungsproblem* which he first encountered as an undergraduate in 1931) in two essays, one written in 1966 for the *American Scientist*, and the other eight years later in a symposium on the philosophy of Karl Popper.

Opinions about this book will depend upon the spirit in which it is read. It tackles the most intractable of all philosophical problems: the relation between objective truth as a scientist sees it and subjective consciousness as a man feels it in himself. Those who look for a solution to this problem will, of course, not find it. Those who relish every thoughtful re-examination of this problem will appreciate the elegance with which it is presented. In brief, it is a book to be read not so much for instruction as for delight. □

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Point defects in solids

Defects in the Alkaline Earth Oxides. By B. Henderson and J. E. Wertz. Pp. 152 (Taylor and Francis: London, 1977.) £8.50.

THIS monograph is concerned mainly with the structure and properties of point defects studied primarily by the techniques of optical and paramagnetic resonance spectroscopy, a field of investigation to which the authors have themselves made significant contributions during its development over a period of nearly 20 years.

MgO and CaO have been extensively studied, the other oxides less so. Transition metal impurities occupy substitutional sites, sometimes in association with nearby impurity or defect sites, and detailed tabulations of data are given. Whereas early studies were often complicated by the unwanted presence of impurities, it has much more recently become apparent that impurities can play an important part in moderating the production of lattice defects by particle irradiation. The structure of anion vacancy defects

seems to be very well understood, although the interpretation of observations relating to cation vacancy defects still presents some problems. The power of double resonance techniques, both ENDOR and those involving simultaneous irradiation at optical and microwave frequencies, is beautifully illustrated. Compared with the extensive array of experimental results on anion and cation vacancy defects, the information available about point interstitial defects is still sparse.

The appearance of this review is timely as the subject has reached that stage of maturity at which a perspective view of the considerable amount of published work is of real value both as a summary of achievement and as an aid in directing attention to the nature of the most significant problems which remain to be solved. The selection of material is well balanced and concisely presented, and will serve not only as a useful compendium for those engaged in this field but also as a helpful guide and introduction for the newcomer.

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Electrochemical techniques

Electrochemistry of Biological Molecules. By Glenn Dryhurst. Pp. xii+601. (Academic: New York and London, 1977.) \$47; £33.35.

POLAROGRAPHY was invented in 1922 and soon became an important method of chemical analysis. At the end of the 1950s, there was a decline in the practical everyday use of this method, because direct current (d.c.) polarography, particularly its sensitivity, no longer met the demands then being made on modern analytical methods. Recent years have seen, however, a renaissance in polarographic and voltammetric analyses, and from classical d.c. polarography a number of modern electrochemical techniques have been derived. Of these, derivative (differential) pulse polarography has surpassed d.c. polarography in sensitivity by about two orders of magnitude, and has become increasingly applied to biological research. The publication of Dryhurst's book is therefore timely.

The content of this book covers a narrower research area than that suggested in the title. Of the compounds with biological significance, only nitrogen heterocycles are included. Electrochemistry is mainly limited to the

studies of these substances using polarography and related techniques. The introductory chapter summarises the theory and instrumentation of electro-analytical measurements in a way that will be easily understood even by readers with no experience of electrochemistry. Further chapters contain information on electrochemical reduction and/or oxidation of N-heterocycles, including purines, pyrimidines, pteridines, flavines, pyrroles, porphyrines and pyridines.

About 250 pages are given over to nucleic acids and their constituents. Although the electrochemical reduction of nucleic acid constituents and their analogues has already been studied in considerable detail (there is also quite a vast literature on purine oxidation), more detailed research on the electrochemical behaviour of oligonucleotides is still needed, representing the missing link between studies of polynucleotides and their monomeric units. Nevertheless, research on nucleic acid structure and properties using electrochemical analysis has developed rapidly in recent years. A correlation between the reducibility of nucleic acids in solution and their conformation is the basis for the application of polarographic techniques to the investigation of small changes in DNA double-helical structure. Changes in nucleic acid reducibility can also serve as an indication of conformational changes occurring in nucleic acids adsorbed at interfaces.

On the other hand, literature concerning oxidation of polynucleotides is as yet practically non-existent.

Included in the book are more than 1100 references, from which an impressive amount of electrochemical data has been extracted. This monograph is an invaluable source of information for electrochemists; it will probably also be useful to biochemists

and biologists interested in properties of biologically important N-heterocycles and their analogues.

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European pollen flora

The North-West European Pollen Flora. Vol. 1. Edited by W. Punt. Pp. 145. (North-Holland: Oxford, New York and Amsterdam, 1977.) \$19.95; Dfl.50.

PALYNOLOGY, the study of pollen grains, is proving to be a valuable technique in a variety of disciplines, particularly environmental ones, both ancient and modern. The recognition of subfossil pollen permits the reconstruction of past vegetation and the detection of palaeo-environmental changes, and the monitoring of present-day pollen fall-out is a necessary part of allergy research. Melissopalynology, the study of pollen in honey, permits the identification of the flowering plants visited by bees.

The rapid growth of these subjects has led to a considerable demand for manuals of palynological techniques and also for data regarding the precise identification of pollen types. At present, the published studies of pollen taxonomic research are widely scattered in the literature and some families have received little attention. This book, and those which will follow it in this series, is designed to remedy this deficiency and to provide an accessible and up-to-date collection of pollen morphological studies. This is being tackled by commissioned review articles which are published in the journal, *Review of Palaeobotany and Palynology*, and which are then collected together in what will be a series of books concerning North-West European pollen.

Obviously, some families are smaller or morphologically less complex than others, so the order in which the published accounts will appear is rather unpredictable. The first collected volume contains accounts of the Caprifoliaceae, Primulaceae, Adoxaceae, Sparganiaceae, Typhaceae, Gentianaceae and Guttiferae. All except for the last of these have been written by members of the Laboratory of Palaeobotany and Palynology of the State University, Utrecht, Netherlands. The account of the Guttiferae is by G. C. S. Clarke of the British Museum (Natural

History).

Each family is divided into a series of "pollen types". The use of the term "type", however, departs from the convention normally adopted in palynological studies, in which "type" is used only when a number of taxa are indistinguishable on the basis of their pollen. Here, a pollen type may contain only a single species which makes the term "type" somewhat redundant. Keys are provided to the pollen types within each family, and full descriptions of the pollen morphology are given. The descriptive terminology of Reitsma is used throughout, which is commendable, for this provides a more consistent and logical system than the earlier ones of Erdtman, and Faegri and Iversen. The descriptions are very full, though it might have been valuable to have added the shorthand designation devised by Iversen and Troels-Smith. Size measurements are given for grains mounted in both glycerol jelly and in silicone oil, which is useful since both of these media are extensively used. One feature which leads to a certain degree of concern is that the descriptions are based upon a very limited number of collections. Some of the types are described on the basis of just one or two collections, and these can hardly be expected to display the full potential range of variation within the taxa.

Excellent collections of photographs are included with each account. These are based upon light field, phase contrast and scanning electron microscopy. They provide extremely valuable supplements to the pollen descriptions. The plates would be far simpler to use if the legends were included on them rather than preceding them. This may seem a minor criticism, but it does limit the speed and efficiency with which the plates can be consulted.

This series will undoubtedly prove to be the definitive work on the pollen of north-west Europe for many decades and one must congratulate the authors, editors and publishers on the production of a most valuable and useful book. I look forward to the appearance of further volumes in this series.

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Marsupial biology

The Biology of Marsupials. Edited by Bernard Stonehouse and Desmond Gilmore. Pp. viii+486 (Macmillan: London, 1977.) £19.50.

It is a measure of current interest in marsupial biology that two substantial volumes with identical titles but different editors and panels of authors have been published on this subject in 1977. The volume edited by Stonehouse and Gilmore is concerned very largely with Australian marsupials, and 28 of the 34 contributors have studied marsupials in Australia or New Zealand. Although the editors' hope that the book "should form an introduction to current research on this long-neglected but fascinating group of mammals" is a laudable one, only about half the contributors have attempted to do this by reviewing their particular field. These chapters are all useful and some are excellent.

Clemens' chapter provides an overview of current theories of marsupial origins and relationships to other therian groups. His conclusion that most land masses could have been inhabited by primitive therians in the early Cretaceous, any one of which could have been the site of origin of marsupials and placentals, is provocative, as it allows for a reversal of the conventional view of origin in North America and a southward spread in the late Cretaceous and Tertiary.

Hayman reviews the field of marsupial karyology from a much firmer data base. The chromosomes of marsupials are better known than for most of the orders of mammals and understanding of the evolution has reached an advanced stage which this chapter reviews.

The functions of the adrenal cortex of marsupials are admirably reviewed by McDonald, while Setchell has written a wide ranging, scholarly account of reproduction in male marsupials, a field hitherto often neglected.

Of the ecological chapters, that by Lee, Bradley and Braithwaite on the resolution of the unique phenomenon of synchronous mortality of male *Antechinus* is fascinating as a piece of problem solving by a coordinated group with several different skills, while Parker's more theoretical chapter, deserves special mention as the first serious attempt to compare the ecological consequences of marsupial and eutherian reproductive strategies. Her conclusion is that the marsupial strategy allows a much slower rate of resource utilisation, first by the mother and subsequently by the slower growing offspring. "Viewed in this context, the

reproductive biology of marsupials cannot be deemed less efficient than that of placentals and may indeed have considerable advantages in the range of environments in which marsupials are found."

This quotation may stand as the theme of the volume itself, for it is a conclusion that is reiterated by many of the contributors whether in relation to physiology, to biochemistry, to ecology or to behaviour.

I have two criticisms for the editors of *The Biology of Marsupials*. The first is that almost half the chapters are reports of hitherto unpublished work more appropriate to a journal, or are narrowly restricted to reviewing an author's own work. A firmer editorial direction to authors would have made the book much more useful and better value to the wider audience. Second, those unfamiliar with marsupials are likely to be confused by the variety of synonyms and common names used in different chapters. The worst example

occurs on p328 where four of the eight species named in Table 19.1 are different from the check-list; and the marsupial mouse *Antechinus swainsonii* is provided with the generic name of the common wombat. Uniform nomenclature throughout the volume could easily have been achieved, since Kirsch and Calaby in their chapter provide a comprehensive check-list of all living species of marsupials.

Despite these shortcomings, *The Biology of Marsupials* provides many reasoned and informed reviews, which will be standard references for some time to come, and which should help to lay to rest the old and now uninformed view that marsupials are intrinsically inferior mammals unworthy of serious study.

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Non-linear acoustics

Theoretical Foundations of Non-Linear Acoustics. By O. V. Rudenko and S. I. Soluyan. Pp. 274 (Plenum: New York, 1977.) \$47.40.

NON-LINEAR ACOUSTICS deals with a very wide range of fascinating phenomena nowadays, including ultrasonic propagation, cavitation in liquids and large amplitude stress waves in solids, as well as the more familiar finite amplitude waves in gases and liquids. Despite the rapidly increasing practical applicability of these phenomena, and despite the review articles, it is a subject which still receives far too little attention in the West, where most of the activity is motivated only by applications to underwater sonar equipment, to propagation in aeroengine ducts or to supersonic aerodynamics.

The Physics Department of Moscow State University, under the late Professor R. V. Khokhlov, has contributed greatly to non-linear acoustics, and has taken a wider view of the subject than any other group. The book under review is by two of Khokhlov's former students, now distinguished in their own right, and is devoted largely to Soviet work. Some of the work is in fact new, and much of it is made accessible to Western readers for the first time in this publication.

The book gives an account of theoretical developments in plane-wave propagation, with diffusion and relaxation, of cylindrical and spherical waves, of sound-sound interaction, acoustic

streaming, the propagation of non-linear beams with lateral diffraction included, and of phenomena occurring in plane waves subject to stochastic influences of various kinds. Of these, the last two are the most novel and repay careful study. Throughout, the authors' aim is to arrive quickly, though often superficially, at a relatively simple governing equation generalising the well-known Burgers' equation for plane flow, which is then solved by *ad hoc* approximate techniques or numerical methods. No attempt is made to use modern asymptotic techniques (such as have been used in Western work on non-linear gas dynamic problems, which are ignored in the references given here). The book therefore loses in the range of problems which can be solved and to some extent in unity of approach. Comparisons with experiment are only made in a generalised fashion.

Despite these criticisms this is a valuable book, and should arouse much interest in the West. The price, however, is high and the translation literal and unidiomatic. More notes from the translator would have helped; readers will search in vain for a description of the "Method of Stageswise Simplifications", and there is much more here in similar vein. But Dr Beyer and the publishers have served us nonetheless by making this book available reasonably quickly after its appearance in the USSR.

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